BIOCHEMICAL AND IMMUNOLOGICAL STUDIES IN PLASMODIUM BERGHEI INFECTED MICE

THESIS SUBMITTED FOR THE DEGREE OF

Doctor of Philosophy

IN

BIOCHEMISTRY

TO

THE FACULTY OF MEDICINE

ALIGARH MUSLIM UNIVERSITY, ALIGARH

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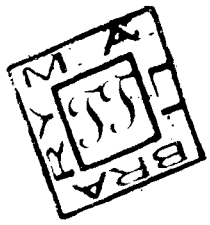

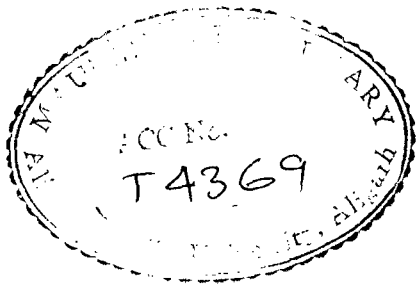

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Dedicated
to
My Family

CERTIFICATE

This is to certify that the research incorporated in the thesis entitled **'Biochemical and Immunological Studies in P. berghei Infected Mice'** has been carried out in my laboratory by Ms. **Neeru Sharma.** I consider this work suitable for submission for the award of **Ph.D. degree** in **Biochemistry** of the Faculty of Medicine, J.N. Medical College, Aligarh Muslim University, Aligarh.

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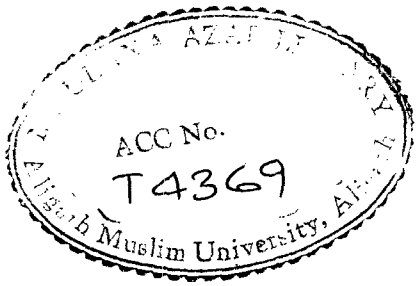

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ABSTRACT

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Malaria continues to be one of the major killer disease inflicting human race in tropical countries. Malarial infection causes structural and functional alteration in different organs of host viz. liver, spleen and kidney. Several attempts have been made at structural and biochemical levels to understand mechanism for liver damage in malaria infected host, since liver is the primary organ of homeostatis in mammals. However its complications remains the prominent cause of morbidity and mortality in the developing world.

In the present study, cell-line of rodent malaria parasite, Plasmodium berghei (NK-65 strain, India) was maintained. The parasite upon inoculation caused 100 percent mortality in white albino mice. The percent parasitaemia was evaluated by Leishman stained thin smears of infected blood, when parasitaemia reached upto 60-70 percent, animals were sacrificed by cervical dislocation, their liver were excised and then homogenized for estimating the different biochemical constituents.

During the infection the total lipid content in liver was increased significantly by (84.49%). The present study showed that liver cholesterol content in albino mice was depleted significantly, similarly phospholipids content of liver was found to decrease by (19.90%). The study further revealed

that liver from P. berghei infected mouse produces more lipid peroxidation as compared to control animals (314%). The effect on carbohydrate metabolism was also indicated by decrease in glucose and glycogen contents by (78.76%), (84.87%) and (78.6%) respectively. As regards to protein metabolism significant decrease in liver protein values was observed, indicating extensive proteolysis of amino acids for proliferation of parasite. Another reason for decrease in protein synthesis might be due to decrease in content of nucleic acid. DNA decreased by (79.58%) and RNA by (29.7%). With the onset of infection, a statistically significant decrease in GPT and GOT level by 22% and 42.5% respectively, were found in liver. Similarly, content of acid and alkaline phosphatases were estimated during severe infection. Acid phosphtase and alkaline phosphatase content of liver during P. berghei infection increased significantly, the acid phosphatase by 75% and alkaline phosphatase by (90%).

On the other hand, the liver damage caused by invading parasites was demonstrated by performing several liver function tests in serum to evaluate the levels of SGPT SGOT acid phosphatase and alkaline phosphatase. SGPT level in serum was found to increase by (188%). SGOT level increased by (84%), acid phosphatase by (215%) and alkaline phosphatase by (99%).

Total lipid, and nucleic acids contents in tissue were also demonstrated by some histochemical technique. Histochemical studies revealed that liver section showed large amount of deposition of lipid droplets throughout the lobules, which mainly causes fatty liver. We have also confirmed decrease in nucleic acid content in infected liver tissue section by above technique.

The role of immunity in protection or at least in minimizing biochemical alteration was studied in immunized animals. Albino mice were immunized against Plasmodium berghei using soluble P. berghei antigen in combination with TDM (6'-6' Trehalose Dimycolate). The intracellular parasite, P. berghei was isolated from infected RBC by means of multistep experimental protocol. The infected blood was collected in ACD (Acid citrate dextrose), 85 percent platelets and 98.9 percent leucocytes were removed by passing the infected blood through column packed with C-cellulose and microcrystalline cellulose.

Saponin was successfully used to induce the lysis of red blood cells. About 100% erythrocytes were lysed and the RBC debris was removed by washing with chilled normal saline. Parasites were separated from this lysate by density gradient centrifugation on Histopaque. The parasites appearing in a brown band at interface were withdrawn carefully and washed three times with normal saline (0.154M NaCl, pH 7.2). The purity of isolated parasite preparation was checked microscopically.

There were no apparent host cell contaminants in Leishman stained smears prepared from isolated parasite material. For the preparation of antigen, the isolated parasites were disrupted by ultrasonication at 9KHz for 6 mts. This preparation centrifuged at 10,000 rpm for 1 hour. The supernatant thus obtained was used as soluble antigen in these investigation.

The purity of isolated P. berghei antigen was checked by immunodiffusion (ID), counter immuno-electrophoresis (CIE) and PAGE analysis. Nonwithstanding the host cell contaminants, It was assumed that the isolated antigen preparation were comparatively pure by using above techniques.

Concentration of protein, DNA, RNA and hexoses in soluble antigen extracts was estimated as 2,500 ug, 200 ug, 400 ug and 200 ug respectively. Antigen characterization was also carried out on sodium dodecyl polyacryl amide gel electrophoresis. The parasite antigen extracts resolved into eleven protein bands in the molecular weight range of 12,000 to 150,000 daltons.

In the immunized animals, total leucocyte count was found to increase. The increase in total leucocyte count was highest in animals immunized with Ag plus TDM. Biochemical assays were carried out in animals after completion of immunization. Serum transaminase, phasphatase contents of

albinomice after immunization were found nearer to control values.

Inoculation of 500 ug TDM alone also provide 100% protection by providing non-specific resistance in host against the infection.

Detection of humoral immune response was carried out by immunodiffusion, indirect haemagglutination (IHA) tests and enzyme linked immunosorbant assays (ELISA). Animals which were immunized with Ag-TDM preparation showed precipitin bands in ID test. Highest reciprocal titre of 512 by IHA and 1024 by ELISA tests was detected in animals which were immunized with Ag-TDM combination. Specific antibody titre values were comparatively lower in animals immunized with antigen and adjuvant alone.

The cell mediated immune response in the immunized animal were detected by delayed type skin hypersensitivity (DTH) reaction and leucocyte migration inhibition (LMIT) test. The delayed type skin reaction reached a maximum at around 24-48 hours in animal immunized with Ag-TDM combination. Other experimental group showed weak skin reaction indicating the absence of proper CMI response. The migration of peritoneal exudate cells obtained from the animals immunized with Ag-TDM was greatly inhibited in presence of P. berghei antigen. There was no significant

migration inhibition of cells from other control groups.

Further, histopathological studies revealed that the liver parenchyma showed patchy necrosis in centrilobular region indicating hepatocellular damage. Sinusoids were dilated, filled with hypertrophied kupffer cells containing phagocytosed parasites, malarial and hemosiderin pigments.

Immunized animals did not show any parasitic infiltration in liver. No pathophysiological changes were observed in immunized liver sections. Haematoxylin and eosin stained sections of liver from immunized animals showed normal architecture.

The findings made from various experiment has helped in understanding the aetiology of the massive changes that takes place at high parasitaemia, in mice during P. berghei infection. The immunological studies further showed that immunization against malarial infection helps in minimizing morbidity and mortality due to P. berghei infection.

1.1 HISTORICAL REVIEW

Malaria still constitutes a continuous threat to almost half of the world's population. It is the most important communicable disease caused by sporozoan parasites of the genus, Plasmodium, causing substantial mortality. Human malaria is probably as old as mankind. As legend or history makes us believe, prehistoric man in the old world was victimized by malaria. Historically, the description in Edwin Smith's Surgical Papyrus, in 1600 B.C. (Breasted, 1930), associated malaria with contaminated air. In Papyrus Ebers, 1550 B.C. (Garnham, 1966), the symptoms of malaria initially described were: fever, rigour and splenomegaly. An account of malaria as it occurred in Egypt was later given by Halawani and Shawrby (1957).

Hippcrates, in 400 B.C. (Boyd 1949), gave the first accurate clinical description of malaria. He had for the first time mentioned about the classic triad of chill, fever and sweating. He further analyzed the characteristic periodictity of various forms of malaria and the associated splenomegaly with the endemicity of malaria and its topographical aspects. Many Roman historians and writers have mentioned about certain fevers, especially those which affected human population in the vicinity of marshes (Boyd, 1949), Celsus, in the first century A.D., gave a rather precise description

of a febrile disease from which falciparum, vivax and quartan malaria could be easily identified as separate entities. Malaria fevers were also known in ancient China, India and Arabia.

Early attempts to prevent what appears to have been malaria are also contained in Edwin's Surgical Papyrus, (Breasted, 1930). During the middle of the seventeenth century, came the first account of the clinical treatment of malaria with the bark of a Peruvian tree, Chinchona. The bark was employed in local Indian medicine, as a febrifuge, although its use was quite limited (Jarcho, 1964). Pelletier and Caventon in 1820, for the first time succeeded in extracting two alkaloid which they named quinine and cinchonine. Out of the two quinine was found to exert a better antimalarial effect.

But more important events in the history of malaria research took place only towards the end of the nineteenth century. In 1880,, Laveran first saw and described malaria parasite in the blood cells of man. Romanowsky (1890, 1891) in Russia developed a new method of staining malaria parasites in blood films. The development of staining techniques, along with later improvements in microscope designing made the study of plasmodia much simpler. Gerhardt (1884) showed the induction of malaria in healthy persons

through inoculation of blood from malaria patients. These studies and those on the morphological aspects of blood schizogony by Marchiafava and Celli (1884), and ultimately the observation on fertilization of a macrogamete by a microgamete (Mac Callum, 1897), finally dispelled the myth of a miasmatic origin of malaria, as also it helped in discarding other rather adventurous hypothesis of the various far-flung causes of the disease. Infact, the way in which malaria was transmitted from man to man remained a mystery for a very long time, although the association between mosquitoes and malaria and the existence of a particular mechani sm of transmission were long suspected. Pfeiffer, in 1892, suggested that malaria parasites passed through an exogenous cycle in the body of a blood sucking insect. Manson (1894) discovered that mosquitoes act as arthropod hosts for malaria parasites. Ross (1897) described gametocyte maturation of P. falciparum in Culex and Aedes and the production of oocysts in anopheline mosquitoes.

Bignami (1899) succeeded in infecting a healthy volunteer with P. falciparum through mosquito bites and elucidated the sporogony cycle of P. falciparum and P. vivax in anopheline mosquitoes (Bastianelli and Bignami, 1899).

1.2 HUMAN MALARIA STRAINS

Over one hundred different species of Plasmodia

are already known to cause malaria in a wide range of vertebrates, each one of them exhibiting a narrowly defined host specificity. Out of the several species, only P. falciparum, P. vivax, P. malariae and P. ovale are naturally infective for man. Amongst the four human species, P. falciparum is the most lethal form of parasite causing malignant tertian malaria in man. Amongst the many other species of Plasmodia, P. knowlesi is reported to cause natural human infection in Malaysia (Chin et al., 1965). Similarly, a doubtful human infection with P. simium is reported from Brazil (Deane et al., 1966). Some accidental laboratory infections are also traced to have been caused by P. cynomolgi (Coatney, 1979).

1.3 RODENT MALARIA PARASITE - PLASMODIUM BERGHEI

Till the later part of 1940, research was mainly carried out using avian malaria and to a lesser extent with simian malaria parasites. In 1948 malaria parasites were discovered in small rodents. These parasites were found to be infective in mice and rats. Since the first isolation and description of P. berghei, malaria parasites from infected murine rodents have been collected from several widely separated regions of tropical Africa (Vincke and Lips, 1948). Thus, a malaria parasite, P. berghei, became available which would infect the cheapest, most suceptable and easily maintained laboratory animals, the mouse.

Various species and sub-species of rodent malaria exhibit different degrees of virulence in unnatural hosts. The

virulence also greatly depends on the age and strains of the host. The mortality rate due to P. berghei infection varies with different strains of mice and young rats. These parasites often run a fulminating and fatal course of infection in certain hosts (Carter and Diggs, 1977). In blood induced infection, parasitemia may reach up to 80-90% within 5-6 days. In most resistant strains of mice and in adult rats, the course of infection is entirely different. The age of the host is often important, in determining the susceptibility of an animal. Adult rats (more than 7 & 8 weeks old) are normally seen to survive P. berghei infection (Singer et al., 1955). Similarly, adult rabbits are refractory to infection with P. berghei but new born rabbits develop parasitemia which may go as high as 5 percent (Garnham, 1966).

Rodent malaria often provides a very convenient and suitable model system for studying certain aspects of human malaria. The discovery of P. berghei led to the establishment of new species of malaria in rodents. Subsequent to its discovery P. berghei parasites have rapidly become a very useful experimental tool for research in parasitology, immunology, biochemistry and chemotherapy of malaria.

1.4 HOST PARASITE RELATIONSHIP

Several recent studies have shown that host factors may modulate the expression of parasite antigens during

malarial infection. These factors include several host-parasite interactions such as immunity, genetic factors, age, nutritional status of host, exposure to the mosquito bites, etc. The susceptability of the host is usually greatest in the young animals. The primary requirement for blood stage infectivity is the presence of a specific red cell receptor for merozoite attachment (Miller et al., 1973). In case of P. knowlesi and P. vivax this receptor is associated with duffy blood group determinants (Mason et al., 1977). The presence or absence of the immune system, or the organs responsible for it, affects the course of infection and the susceptibility of the host as well. For example, absence of spleen can convert a resistant host into a susceptable one, or low grade parasitemia can be converted into a fulminating high grade parasitaemia (Garnham, 1970). Absence of Para-aminobenzoic acid in the host diet renders it more resistant to infection. Similarly, several intraerythrocytic factors also influence parasite development and contribute to innate resistance. Amongst these are structural modifications in haemoglobin (especially Hbs), quantitative changes in haemoglobin chain synthesis (thalassemia), Glucose 6-phosphate dehydrogenase deficiency and levels of ATP. The inhibitory effect of sickle cell haemoglobin upon P. falciparum has also been observed by Friedman (1979).

1.5 LIFE CYCLE

The life cycle patterns of plasmodia in non-human primates, rodents, birds and reptiles were reviewed by Collins and Aikawa (1977), Carter and Diggs (1977), Seed and Manwell (1977) and Ayala (1977). Malaria parasite has got a complex life cycle alternating between vertebrate and arthropod hosts. Through the bite of a female anophelene mosquito the infective sporozoites are inoculated into the blood stream of a vertebrate host. The sporozoites then enter into the hepatic cells of the host, where drastic morphological changes follow. First of all, the parasite appears round or oval and contains a chromatin nucleus surrounded by cytoplasm. During this exo-erythrocytic schizogony, the nucleus divides and the cytoplasmic mass grows leading to the formation of schizonts. After this, the schizont segregates and the merozoits are formed and released. The released merozoites enter the blood circulation and invade the red blood cells. In the erythrocytes, the rapid changes continue to take place. The ring stage parasites are the first to appear, this is followed by trophozoite and shizont stages. The mature schizont bursts, liberating the merozoites and lysing the red blood cells. The merozoites so released again attack new RBCs to continue the cycle. During this process the haemoglobin in digested by the parasite, it also produces a typical malarial pigment the

hemozoin. Apart from the blood schizogony, the merozoites can also develop into microgametocytes (male gametocyte) and macrogametocytes (female gametocyte). When taken up by a suitable arthropod host, the gametocytes transform themselves into male and female gametes. The gametes after fertilization, sequentially proceed to form the zygote, ookinete, oocysts and sporoblast to give rise to countless new sporozoites in the body cavity of the insect vector.

1.6 PATHOLOGY

Even before the discovery of the causative agent of malaria, the presence of a characteristic brownish pigment in the spleen, liver, kidney and brain was noted by most pathologists conducting necropsies on people who had died of this affliction. In 1847, Meckel pointed out that the brown colouration of these organs was caused by the accumulation of pigment removed from the blood.

Pathological changes in malaria result primarily from the infection of erythocytes by Plasmodium and from the host's response. To understand these changes three factors contributing to the development of pathological lesions in various organs, must be considered: (1) parasitaemia, (2) destruction of damaged erythrocytes, and (3) the host's defence response against the infection, including phagocytosis and the

development of immunity. Malarial parasites invading erythrocytes initiates pathological process, and the consequences of this infection influence the host's other tissues and organs (Mason Bahr and Apted, 1982). Destruction of host red blood cells occurs not only when plasmodia cause rupturing of the erythrocytes at the end of schizogony but also through phagocytosis of infected and uninfected erythrocytes.

1.6.1 RENAL PATHOLOGY

There are only few studies on histopathological changes in the kidneys of rodents infected with malaria (Miller et al., 1968). Greenwood and Voller (1979a) were the first workers to report the histopathological changes in kidney of mice infected with P. berghei. Ehrich and Voller (1972) conducted their studies on Swiss albino mice infected with P. berghei yoelii strain 17 X. They observed the deposition of glomerular immunoglobulin with parallel development of parasitaemia. Suzuki (1974) was able to produce an immune complex disease in mice infected with P. berghei. Similarly, George et al. (1976) detected glomerular deposits of IgG and IgM.

Boonpucknavig et al. (1972), in a study on mice infected with P. berghei found antigen deposition along the glomerular capillary walls in a granular pattern, extending

into the mesangial areas. Histologically, the glomerular lesion consisted of mesangial cell proliferation, endothelial cell hypertrophy, polymorphonuclear cell infiltrations and a thickening of glomerular basement membrane. An accumulation of hemosiderin pigment was also observed in the proximal tubular cell.

1.6.2 SPLEEN PATHOLOGY

The spleen is one such organ which shows some of the earliest changes in individuals with malaria. Spleen enlargement is a well known physical sign of infection and its enlargement rate in human populations was used for the evaluation of malaria prevalance within a region (Boyd, 1949). Changes in spleen size in experimental, non-human primates with malaria were evaluated by Coggeshall (1937) who demonstrated that Macaca rhesus infected with P. knowlesi and dying in 3 to 7 days after infection showed a 57% average increase in organ size. If infection lasted longer because of treatment, the increase was 91%. Jervis et al. (1972) working with P. falciparum infected Aotus found the spleen of infected monkeys to be larger and heavier than those of uninfected control monkeys. The anaemia seen in malarial infections of humans and animals can sometimes be profound, and extramedullary erythropoiesis is often seen in the spleen.

Singer (1954) found that after seven days of P. berghei infection, the chief activity of the spleen of mice was erythropoiesis. The tropical splenomegaly syndrome (Pitney, 1968) may be a most important consequence of malaria.

1.6.3 LIVER PATHOLOGY

Liver is the most important and the largest gland in the mammalian body. Bloom and Fawcett (1980) have stated that it functions both, as an exocrine as well as an endocrine gland. It is also one of the main organs for homeostatis. All the absorbed products of digestion are taken up and metabolized here. A number of drug metabolizing enzymes are always present in liver. Liver may also receive some toxic substances, produced either chemically or through parasite infection.

In most of the pathological situations including parasitic infections, where host's adaptability and homeostasis are altered, liver seems to be one of the most important organs where functions are impaired. Initially the pathological stress produces a very subtle change, which can be detected by means of a few sensitive immunological and biochemical tools as also in light microscopy.

Hepatomegaly is another common sign of malaria infection in humans, though variable in experimental animals.

Jervis et al. (1972) in P. falciparum infected Aotus did not find any significant alteration in the wet or dry weight of liver. But the wet weight in animals infected with P. berghei parasites was increased, while the dry weight remained unchanged, thereby indicating that increase was only due to oedema (Jervis et al., 1968). Taliaferro & Mulligen (1937) have shown that P. cathemerium produces a marked liver enlargement in canaries especially during the crisis and shortly there after. Another significant liver abnormality is the progressive change in its colour from pink to dark brown, and it is confirmed that, after a prolonged infection, the colour of the liver becomes black. This is attributed to the deposition of malarial pigments in the reticuloendothelial system (RES) (Aikawa, 1980).

Liver biopsy studies in human malaria subjects showed Kupffer cell hyperreactivity, production of malaria pigment and variable hepatocellular necrosis at maximum parasitemia (Brito et al., 1962). Swelling of hepatocytes, fatty infiltration, centrilobular and midzonal necrosis of hepatocytes has also been reported (Maegraith, 1958). Fulton et al. (1953) have noted in mice infected with P. vinckei an increased phagocytic reactivity of reticuloendothetial system. Chow and Kreier (1972) have also observed in mice infected with P. berghei malaria that kupffer cells become hyperactive towards

the phagocytosis of malarial pigment, parasitized erythrocytes and other cellular debris.

By light microscopy, many workers have demonstrated profound changes in the reticuloendothelial system. It is generally accepted that the endothelial cell lining of liver sinusoids are phagocytic in nature and they can transform into Kupffer cells (Aikawa et al., 1968). These transformed endothelial cells divide rapidly to increase the number of macrophages. In P. falciparum of humans and experimental monkeys (Jervis et al., 1972; Gutierrez et al., 1976), large number of parasitized red cells have been seen attached to the endothelial cell.

Electron microscopy has shown that kupffer cells are vacuolated and contain electron dense cytoplasmic bodies with large phagolysosomes containing infected erythrocytes and malarial pigment particles (Aikawa and Antonovych, 1964). No apparent digestion of the pigment particles is reported but digestion of parasitized red blood cells along with an acid phosphatase activity has been demonstrated in phagolysosomes (Aikawa et al., 1968). Ultrastructural liver lesions in natural infections of P. falciparum and P. vivax in man have been reported by Rosen et al. (1967) and Brito et al. (1969) respectively.

Maegraith et al. (1962), Fletcher and Maegraith (1962) have reported in hosts as rats, mice and monkeys, which were infected with P. berghei, P. Yoelii, P. knowlesi and P. falciparum, that at higher parasitemia a well marked swelling occurs of the liver mitochondria, along with a disorganization of their cristae, and finally the vacuolization of the matrix. Maegraith (1966) have noticed events such as: lipid infiltration, glycogen depletion in centrilobular cells, loading of kupffer cells, centrilobular necrosis and degeneration of liver cells.

1.7 BIOCHEMICAL CHANGES

1.7.1 MOUSE LIVER

In spite of the enormous amounts of literature on malaria, comparatively little information is available on parasite induced changes in host liver, since the liver tissues is the first and foremost target organ of malarial parasite. Infact parasites derive their nutrition from the host material and liberate metabolic end products in the host system. Up to certain limit no deleterious effects become manifest in the host, though host immune system does get stimulated. When the balance breaks down ill effects of parasitism is observed in the form of pathological lesions and nutritional deficiencies. Significant changes in biochemical parameters occur comprising of different enzymes and chemical constituents of liver.

Common histological feature of the liver tissue in malaria is that of the so called "fatty degeneration", in addition to centrilobular necrosis. Riley and Maegraith (1962) who biochemically examined this aspect found a total increase in the liver fats from 10-25% over control values. The findings by gas chromatography of the fatty acid contents of these lipids supported the above observations, besides showing a significant increase in unsaturated fatty acids such as oleic acid and linolenic acids along with corresponding decrease in the saturated palmitic and steric acids. A similar pattern was observed both in monkeys and mice. In electron microscopy, simple lipid deposition in the form of droplets in close proximity to the damaged mitochondria was a common finding in liver from infected animals (Fletcher, 1964). Gutierrez et al., (1976) have demonstrated increase in lipid contents of liver in Aotus monkeys infected with P. falciparum. Maegraith (1966) has also observed significant increase in lipid content of liver of rhesus monkeys infected with P. knowlesi. Mercado and Von Brand (1958) and Angus (1971) on histochemical examination reported the presence of lipid in the liver of mice and monkeys infected with P. knowlesi and P. berghei, respectively. Further, Rajvir et al. (1981), Saxena et al. (1981), Fletcher (1987) and Gupta (1988) have also found raised levels of total lipid contents. Similarly, Seshadri et al. (1981) have also observed increase in total

lipids during P. vivax malaria. Rao et al. (1967, 1969) and Seshadri et al. (1983) have also observed a massive increase in total lipids and a decrease in cholesterol and phospholipid contents of liver of mice following P. berghei infection. A close relationship exists between cellular injury, peroxidation of membrane lipids and oxidative damage to the liver cells (Sharma and Krishnamurti, 1976). Saxena et al. (1981) have shown that liver and spleen produce higher amounts of lipid peroxidation due to increased susceptibility of these tissues to oxidative damage under the stress of a malarial infection. Chauhan et al. (1981) have also reported increase in the level of lipid peroxides in various tissues of mice infected with P. berghei . Recently, Clark et al. (1987) have observed increase level of lipid peroxidation in human liver during P. falciparum infection. Mahdi and Ahmad (1989) have reported an increase in the rate of lipid peroxidation in P. berghei infected mice brain.

Hypoglycaemia in malarial infections has also been occasionally described (Maegraith, 1948). Fulton (1939) had examined the problem in P. knowlesi malaria in rhesus monkeys and reported hypoglycaemia and depleted liver glycogen in the late stages of infection. Mercado and Brand. (1957), Chatterjee and Gupta (1957) and Devakul and Maegraith (1958) have observed decrease in tissue glucose and glycogen of rats infected with P. berghei, and monkeys infected with

P. knowlesi. In such cases blood sugar level was also found decreased (Saxena et al., 1981). Similarly, Wellde et al. (1972) have revealed decreased glucose content of liver in rhesus monkeys infected with P. falciparum. Srivastava et al. (1984) have reported that during acute P. knowlesi infection in rhesus monkeys, glycogen was depleted for a considerable extent. This depletion was mainly due to excessive breakdown of glycogen by the polysaccharide degrading enzyme, glycogen phosphorylase. Further, Geofforion et al. (1985) have also reported reduced activity of hepatic gluconeogenesis in P. berghei infected mice.

However, White et al. (1983) and Pandey et al. (1986) reported that hypoglycaemia was not uncommon complication of severe malaria. White et al. (1986) have also shown that hypoglycaemia is frequently associated with severe malaria and high parasitaemia In the aetiology of hypoglycaemia in severe malaria, the possibility of monokines acting as significant mediators can not, however, be ruled out (Clark et al., 1981). Clark and his co-workers have produced experimental evidence which according to them indicates a central role for these monokines. In malarial infection of rodents, they have shown a blocking of the induction of enzymes required for gluconeogenesis, a function normally promoted by glucocorticoids, Hypoglycaemia was also observed in humans during malarial infection (Phillips, 1989).

With regards to protein metabolism, significant decrease was reported in liver protein contents which mainly causes hypoproteinaemia. Von Brand (1973) has noted decrease rate of protein synthesis during malaria. Decreased rate of protein synthesis during malarial infection is also reported in mice by Fern et al. (1985). In our studies we have also carried out protein estimation in liver homogenate. It was found to be decreased. This might be due to decrease in synthesis of nucleic acid. But Pandey et al. (1986) have not observed any marked change in RNA and DNA contents in liver, whereas it is depleted significantly in kidneys and spleen.

1.7.2 SERUM ANALYSIS

It is well documented that malaria infection impairs liver function (Riley and Maegraith, 1961). The ultrastructural and biochemical changes in the infected liver cells are, in fact, initiated during the erythrocytic phase through mediators released in serum (Maegraith, 1966). The liver damage caused by invading parasites during acute malaria can be more conveniently detected by means of several routine tests. Sadun et al. (1965, 1966) have observed a significant increase in transaminases (viz. SGPT and SGOT) levels in serum, which is indicative of abnormal liver function during malaria infection. Lal and Hussain (1978) have reported a significant decrease in GPT and GOT activity in liver of mice infected

with P. berghei. Desowitz et al. (1967) have observed an abnormal elevation in SGOT, SGPT, values and a rapid increase in serum alkaline phosphatase activity in rhesus monkeys. Similarly, Seshadri et al. (1981) observed a significant increase in SGPT and SGOT during P. vivax malaria. Further, Saxena et al. (1981) and Srivastava et al. (1982) and Khanna et al. (1986) have reported significant increase in SGOT, SGPT value in infected animals.

1.8 IMMUNOLOGICAL STUDIES

1.8.1 ANTIGEN ISOLATION

Malaria parasite is a complex eucaryotic organism. The isolation of malarial parasite in its pure form is rather a difficult task because of intracellular nature of parasite. The parasite material is usually contaminated by host erythrocytic material. In order to harvest intracellular parasites, it is necessary to disrupt the host cell and achieve separation of parasites from the host cell constituents by causing minimal damage to the parasite.

A most economical and convenient source of parasitized erythrocytes for laboratory study, is the blood of rodents infected with one of various rodent plasmodia.

(A) Preparation of Parasitized Erythrocytes

Blood collected from infected animals, or humans, which is to be used for harvesting plasmodia, may be subjected to certain preliminary treatments to remove unwanted blood components, and to increase proportion of parasitized erythrocytes. An integral part of such a treatment is the removal of plasma by centrifugation and washing. The buffy coat may be removed from the packed red cell mass, after centrifugation, for reducing the number of platelets and leucocytes (Zuckerman et al., 1967). Leucocytes are also removed by filtration of infected blood through a millipore filter of 5 um pore size (Brown et al., 1966). Leucocytes may be removed by suspending the washed blood cells in several volumes of dextran solution and allowing the mixture to sediment in graduated cylinder. The leucocytes which are left in the suspension are then discarded (Langer et al., 1967). Washed blood cells on a 10% dextran solution (Mw 50,000) have also been employed for removal of leucocytes (Levy and Chow 1973).

The passage of the diluted blood through a column of filter paper powder was also used for removing leucocytes (Homewood and Neame, 1976). Leucocytes may be removed by passing whole blood through a column, made up of a mixture of equal amount of SE cellulose and Sephadex G-25 (Nakao,

Nakayama and Kankura, 1973). The passage of whole blood through a column made up of equal parts, by weight, of ∝-cellulose and microcrystalline cellulose provides an efficient means for removal of all the leucocytes and most of the platelets from blood (Beutler et al., 1976).

(B) Release of Parasites from Parasitized Erythrocytes

The isolation of malarial parasites is one of the most crucial step in conducting biochemical and immunological research. Various physical, chemical and immunological methods are in use to release the parasites from infected erythrocytes (Hamberger and Kreier, 1980).

Christopher and Fulton (1939) introduced the use of saponin for lysing erythrocytes. Siddiqui et al. (1978 b) showed that saponin was the most effective substance for hemolysis and for the removal of membrane proteins. Prior et al. (1973) used 0.83% ammonium chloride solution buffered to pH 7.4 with 0.17 M tris buffer to release plasmodia from the host cell. Haemolytic antiserum along with complement has been used by various workers for the isolation of parasites from infected erythrocytes (Langer et al., 1967; Trigg et al., 1975).

Coleman et al. (1979) have used heat stable hemolysin produced by Pseudomonas aeruginosa for the lysis

of P. berghei infected erythrocytes. Verain and Verain (1956) used ultrasound to disrupt the red cells but it was found to cause damage to the parasites (Prior and Kreier, 1977). This problem was resolved by development of continuous flow system (Prior and Kreier, 1972 a,b).

(C) Separation of Freed Parasites

Heidrich et al. (1979) have described the usefulness of free flow electrophoresis for separating parasites from host erythrocyte fragments. Similarly, differential and density gradient centrifugation have been used for purifying parasite preparation by Prior and Kreier (1972 a) and Eisen (1977). Siddiqui et al. (1987) employed a three step gradient prepared from 37%, 23% and 10% sucrose solutions with low speed centrifugation for obtaining freed. P. falciparum parasites.

The Histopaque density gradient yields a comparatively more defined product (Kumar and Ahmad, 1983) than those described earlier by Prior and Kreier (1972 a) and Heidrich et al. (1979). In the present study parasite preparations were purified using density gradient centrifugation on Histopaque solution.

(D) Disruption of Parasite

The isolation of antigen involves the disruption of collected parasites and separation of soluble material from

the cell debris. A great variety of techniques have been applied. These include: lysis by distilled water (Cook et al., 1971), use of Triton X-100 (Sherman et al., 1975), Freezing and thawing (Sherman, 1964) and sonication (Todorovic et al., 1968). Treatment with French pressure cell (D'Antonio, 1972) or Huge Press (Spira and Zuckerman, 1962) has also been applied along with homogenization (Jerusalem, 1971).

1.8.2 ANTIGEN CHARACTERIZATION

Soluble extract of plasmodial organisms usually consists of a complex of potentially antigenic components. Various procedures have been used for fractionating antigens. These antigen fractions have been tested in double immunodiffusion, immunoelectrophoresis and ELISA, for identification of plasmodial extracts. The various procedures used for fractionation of antigen are: analytical ultracentrifugation, sucrose density gradient ultracentrifugation, gel filtration on sephadex G-100 and G-200, ion-exchange chromatography on DEAE cellulose or on CM-cellulose and block electrophoresis with starch, or with Pevikan Gehon (Chavin, 1966). But none of the above procedure provides a really suitable method for fractionation of plasmodial antigen. Ion exchange chromatography yields upto three fractions, while other procedures yield only one fraction. Mannweiler and Oelerich (1969) fractionated the extracts obtained by prior freezing

and thawing of saponin released P. berghei by gel-filtration and preparative PAGE. The antigenic activity of different fractions has also been checked in double immunodiffusion tests. (Hamberger and Zuckerman, 1976).

The soluble extracts of primates, rodent and avian plasmodia resolved into a number of potentially antigenic components on PAGE (Chavin, 1966; Grothaus and Kreier, 1980). For human plasmodia, P. falciparum antigens have been most extensively studied. The antigens of P. falciparum were obtained by direct lysis of parasitized erythrocytes. In these studies antisera from human patients were used for such analysis.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most important method for fractionating soluble extracts of plasmodia. The principle of this technique is based on the presence of a number of polypeptide chains and their varying molecular weights, respectively. Grothaus et al. (1984), who used SDS-PAGE for fractionation of P. berghei have found a number of potentially antigenic components, which may be important in the induction of immunity. Kumar and Ahmad (1984) have also used a similar technique for fractionation of P. berghei extracts. They isolated, on SDS-PAGE, 10 fractions in the molecular weight range between 7,000 to 140,000. Antigenic analysis of P.

knowlesi using electrophoretic technique (Singh et al., 1986) was found helpful in identifying protective antigens, as well as those antigens which were important in the serodiagnosis of human malaria. Characterization of P. chabaudi antigens present on the membrane of RBC infected with ring stage parasites was also carried out (Wunderlich et al., 1987). Recently, Sharma et al. (1990) have carried out purification and characterization of P. berahei isolated antigens on SDS-PAGE. They obtained 11 protein bands. Some of them, hopefully, can subsequently be exploited for their ultimate use in vaccination studies.

1.8.3. ADJUVANT

It is becoming widely recognized that development of successful malaria vaccine will depend upon the availability of a safe and effective adjuvant. After all, the ultimate aim of any vaccine research is to immunize and protect man. Therefore, the search for an immunologically acceptable adjuvant is very important for the development of effective and functional human malaria vaccine. So far, numerous adjuvants have been used in combination with malarial antigens for immunization purposes.

Freund et al. (1948) was the first to demonstrate the importance of using Freund's complete adjuvant (FCA) in

combination with parasite antigen material, for achieving protective immunity. Subsequent vaccination studies in avian, rodent and simian malaria further confirmed the above findings (Cohen et al., 1985; Maheshwari et al., 1989). Many early investigators working with P. knowlesi antigen used FCA in their vaccination studies (Freunds et al., 1948; Targett and Fulton, 1965). The successful immunization of monkeys against P. falciparum antigen was also carried out by several workers (Siddiqui, 1977; Perrin et al., 1984; Siddiqui, 1987 and Holder, 1988).

Although, the FCA is a very effective adjuvant, but it has so many local and systemic side effects. Because of those side effects, FCA is totally unacceptable for human use. Its side effects are so toxic that it cannot be used for veterinary vaccines either.

Recently, alum hydroxide, the only adjuvant approved for human use, along with x-irradiated sporozoites of a mouse malaria parasite were shown to induce protection against sporozoite challenge (Nussenzweig et al., 1967). Similarly, human volunteers immunized with attenuated, X-irradiated sporozoites of P. falciparum were protected against human malaria sporozoite challenge (Clyde et al., 1973). The results of clinical trials with alum adjuvanted sporozoite and merozoite vaccines indicate that such a procedure is safe for use in

humans, having no adverse effects. However, the vaccine itself was found to have a limited immunogenicity and protective success.

Various microbial products, in particular their polysacchridic components, posses the property of enhancing non-specific host resistance, if given prior to the infection. For example, Beta 1-3 glucan, isolated from Saccharomyces cerevisiae, lentinan from Lentinus edodes and Schizophyllum from Schizophyllum commune have all been widely studied for their adjuvant action (Song and DiLuzio, 1979). The ability of glucan to modify resistance and enhance effectiveness of vaccines for microparasitic diseases was extensively studied. Pretreatment with glucan resulted in 80% survival in contrast to the 80% mortality in control mice. Glucan has been widely used for prevention or therapy of toxoplasmosis (Nguyen and Stadtsbaeder, 1980), experimental leprosy (Delville and Jacques, 1980) and Leishmaniasis (Cook et al., 1982). In several other investigations glucan was used as an adjuvant for immunization against P. berghei (Holbrook et al.,1981b, Kumar and Ahmad, 1985 and Maheswari et al., 1989, 1990), Entamoeba histolytica (Ahmad et al., 1980; Sharma et al., 1984 b) and Leishmania donovani (Obaid et al., 1989). It was found that it elicits a very specific protective immune response.

The first well defined immunoactive mycobacterial glycolipid identified, trehalose-6,6'-dimycolate (TDM), was isolated in 1950 by Bloch (Bloch, 1950). Intraperitoneal injection of TDM in oil emulsion was able to protect mice against an intraperitoneal challenge by Salmonella typhi and Salmonella typhimurium (Yarkoni and Bekierkunst, 1976), Oil emulsion of TDM is also active against tumors (Olds et al., 1980; Sakurai et al., 1989). TDM emulsion in the presence of oil is toxic, since the toxicity largely depends on the percentage of oil used (Seqqev et al., 1981). Aqueous emulsions are very stable, they are active in mice against Klebsiella pneumoniae and Listeria monocytogenes (Parant et al., 1977) and can protect mice against Babesia microti (Clark, 1979). Schistosoma mansoni (Olds et al., 1980), Toxoplasma gondii (Masihi et al., 1979) and Trypanosoma cruzi (Leon et al., 1983). Protection studies with TDM were also carried out by Kumar and Ahmad (1984) and Lederer, (1986, 1988) in mouse model system infected with P. berghei. They observed good protection in mice treated with TDM.

Bacillus Calmette-Guerin (BCG) has been widely used in the laboratory and in clinics as an adjuvant for the stimulation of host immunity. Parashar et al. (1982) and Farraroni et al. (1986) noted protection in mice infected with P. berghei, following inoculation of BCG doses in combination with P. berghei antigen.

Another adjuvant, muramyl dipeptide (MDP), was synthesized from whole Tubercle bacilli. This was shown to replace FCA in enhancing the immune response of an animals against a particular antigen when injected with mineral oil (Kotani et al., 1975). Khanna et al. (1991) have demonstrated considerable protection of rhesus monkeys against malarial infection following immunization with P. knowlesi whole antigen in combination with MDP. Similarly protection studies with MDP were also carried out in rat and mice by Khullar et al. (1985, 1988, 1990). Many derivatives of MDP have also been used as an effective adjuvants. It has been demonstrated that stearoyl-MDP (6-0-stearoyl -N-acetyl muramyl -L alanyl -D-isoglutamine) and B-30 MDP could easily be used as a replacement for FCA in effective immunization of owl monkeys against P. falciparum infection (Siddiqui et al., 1978).

Currently, Bathurst et al. (1989) have evaluated the efficiency of MTP, a derivative of MDP, in mice, guinea pigs and rabbits against CS proteins of P. falciparum. Inoculation of B-30-MDP+(LA-15-PH) in combination with antigen provides good protection in mice and rabbits against P. falciparum (Tam et al., 1989; Hui et al., 1990).

1.8.4 VACCINES

Malaria, although infrequent in the developed world, occurs in hundreds and millions of people each year in the

tropical countries and is a potential threat to hundreds and millions of others (Najera, 1989; Siddiqui, 1991). Despite remarkable success of the intensive efforts made against malaria, the disease remains a major health problem of the tropical and subtropical countries. The current resurgence of the disease in Africa, Asia and Central America is increasingly a source of great alarm. In our own country hundreds of people suffer and die of this dreaded parasite disease. For a brief period in early 1970, it appeared that malaria might soon be brought under control. Extensive spraying of DDT greatly helped in reducing anopheline mosquito population, while effective chemotherapeutic measures were equally successful in controlling the disease. A few novel drugs such as chloroquine and mefloquine were successfully used for treating the patients. But ten years later with the resurgence of malaria, the situation appeared entirely different. Logistically, the tools and methods so far available for malaria control did not prove adequate enough to keep pace with the increasing severity of the disease over the past three decades. For instance, the chlorinated hydrocarbon insecticides such as DDT are proving ineffective against anopheline mosquitoes due to the newly acquired phenomenon of insecticidal resistance. Then a more alarming situation had arisen due to resistance of the parasite, P. falciparum, to various antimalarial drugs. Currently, these factors constitute the most important threat towards the efforts

for achieving an effective control of the disease. Chloroquine resistant cases have been detected in various regions of the world, including India, (Marshall, 1990; Prasad et al., 1990).

The failure of conventional measures to control/eradicate malaria from tropical and subtropical areas of the world has ultimately led may scientists to search for a possible malaria vaccine. The development of cultural techniques for in vitro growth of erythrocytic stages of P. falciparum have greatly helped in malaria research after the work of Trager and Jensen (1976) and with the advent of hybridoma technology following Kohler and Milstein's work in 1975 for producing monoclonal antibodies. The goal of developing a malaria vaccine seemed nearer than ever before (Anonymous, 1984).

The malaria parasite undergoes progressive transformation during the course of its life cycle in which the parasite generates an enormous number of antigens. Some of these antigens stimulate protective immune responses of the host, while the others are, immunologically, nonentity or insalubrious (Bruce-Chwatt, 1985). For the production of protective malaria vaccine, the use of antigens which effectively stimulate the protective response of the host against a homologous parasite species is imperative. Several strategies

have been devised for the complex task of selecting antigens that may be important for vaccine production.

Much of the preliminary work has been done in several plasmodial species including P. falciparum and, hopefully, a protective vaccine against human malaria will soon be developed before long. There are numerous candidate vaccines which are currently under investigation. Presently there are three candidate vaccines, a sporozoite vaccine, asexual blood stage vaccine and gametocyte vaccine (Howard et al. 1987; Cattani, 1989).

(A) Sporozoite Vaccine

Although sporozoites remain in body circulation only for a brief period of time before entering hapatocytes, but vaccination of rodents, monkeys and humans with attenuated, x-erradiated sporozoites has provided complete protection against malaria (Miller et al. 1986; WHO, 1990). The main protective antigens of sporozoites are concentrated on its surface. When fully mature the sporozoites within the slivary gland of mosquito are enveloped by a proteinaeous layer, the circumsporozoite (CS) protein. Considerable progress has been made in the study of protective antigens using P. berghei, the murine malaria parasite. The surface of fully differenciated sporozoites of P. berghei is covered uniformly by a major species-specific immunodominant, membrane protein, pb 44, which helps in the

penetration of sporozoite into liver cells. The corresponding purified monoclonal antibody (3 D 11) to pb 44 protein completely protects mice against sporozoite induced infection (Nussenzweig and Nussenzweig, 1984). Egan et al. (1987) tested P. berghei sporozoites as candidate vaccine constructs, which were found to be useful for human malaria vaccine development. Circumsporozoite protein of sporozoite surface of P. berghei contains two specific B and T cells epitopes, which are involved in the stimulation of the immune response. (Romero et al., 1988). These epitopes help in screening of P. berghei genome or CDNA library, in order to identify particular clone sequence, which is mainly involved in the process of sporozoite infectivity (Romero et al., 1989). Moreover, the peptide corresponding to the relevent sequence of P. berghei was synthesized and coupled to a protein carrier with an adjuvant and injected into the animals. Such inoculations induced the synthesis of antipeptide antibodies which neutrilized the infective sporozoites (Laner, 1990).

The most promising achievement with regard to the development of a sporozoite vaccine was gene cloning of P. knowlesi antigens in Escherichia coli. This was done by extracting the messenger RNA encoding circumsporozoite protein from infected mosquitoes through conversion by the enzyme reverse transcriptase into DNA. The fragment of CDNA was inserted into E. coli. The bacterial plasmids were then

introduced into E. coli. The bacterial clones excreted peptides binding to the monoclonol antibodies against circumsporozoite protein of P. knowlesi (Sharma and Godson, 1985; Sharma et al., 1986).

The CS proteins of different plasmodial species possess structural similarities, yet retain the differences which make each species antigenically unique. Each CS protein possess an immunodominant epitope which is repeated several times within the molecule. For P. falciparum, the epitope is situated in an area of some 37 tandemly repeated peptides comprising amino acids (Dame et al., 1984). A 12-amino acid peptide comprising three repeats of the tetrapeptide has been shown to contain the immunodominant epitope, and the antibodies raised against a synthetic peptide were found to react with the surface of sporozoites for neutralizing their infectivity (Zavala et al., 1985 b). Also for P. vivax, the dominant epitope is located in a area of tandemly repeated peptides. In this case, the peptide, comprising nine amino acids is repeated some 19 times (Arnot et al., 1985). Circumsporozoite proteins are immunogenic in man and animals and when sporozoites are in immune serum, the homologous antibodies crosslink with sporozoite surface antigens to give rise to a characteristic circumsporozoite precipitation (CEP) reaction (Vanderberg et al., 1969).

The first synthetic peptide tested in man was safe and stimulated biologically active antibodies (Herrington et al.,

1988; Perlman et al., 1987). Perlman et al. (1988) have also selected immunogen and carried out epitope mapping.

(B) A Sexual Blood Stage Vaccine

The asexual erythrocytic stage vaccine are expected to induce immunity by restricting the replication of asexual blood stage parasites, without necessarily inducing sterile immunity. But consequentially, it is expected to reduce the morbidity and mortality due to malaria.

A number of candidate antigens are being considered for the development of vaccines against the asexual blood stages of malaria parasite. The principle targets for vaccine include antigen on surface of merozoites or mature schizonts, immunization using blood stage antigen derived from avian, rodent and human malaria parasite can induce effective protection in the appropriate host, but the degree of protection varies, depending on the nature of antigen and the form in which it is administrated (Taylor and Siddiqui, 1982). Irradiated parasitized erythrocytes have been extensively utilized as experimental immunogens, some success was also achieved with rodent malaria (Wellde and Sadun, 1967). Zuckerman et al.(1967) also showed some degree of immunity in rats, which were immunized with free extracts of P. berghei both with or without adjuvant. A highly effective immunity in CBS7B1 x Balb CF mice

was demonstrated by Playfair et al. (1977) when they vaccinated their mice with saponin lysed, formalin killed P. yoelii parasitized cells mixed 10^8 pertussis organisms. Khanna et al. (1991) demonstrated considerable protection of rhesus monkeys against P. knowlesi challenge following immunization with whole antigen in combination with an immunomodulator, the MDP. Using a P. falciparum mature schizont vaccine, or extracellular merozoits emulsified in FCA, Siddiqui (1977) obtained 100% protection of immunized owl monkeys following challenge with a homologous strain of P. falciparum. Recently, Siddiqui et al. (1987) have demonstrated protection of Aotus monkeys against malaria using merozoite surface and rhoptry polypeptides, thereby establishing the candidacy of those antigens for a human malaria vaccine. Recently, Perlman et al., 1988 have described the potential of one specific candidate vaccine, RF/55/RESA. Similarly, Patarroyo et al., 1988, reported that some synthetic proteins provide protection in humans. The major merozoite surface antigen of P. falciparum is a 195 kilodalton (KD) protein which is synthesized during schizogony and deposited on the surface of intracellular parasite (Holder et al., 1988). The corresponding purified monoclonal antibodies to 195 KD protein of P. falciparum protect the animal against schizont and merozoite induced infection (Lyon et al., 1987). Successful cloning of P. falciparum blood stage antigen into E. coli through Plasmodial CDNA has also been achieved (Bruce Chwatt, 1986).

Malaria infection induced toxicity is associated with a number of structural, functional and pathological alterations in different organs of host viz, liver, spleen and kidney. The liver is the primary organ of homeostasis and it plays an important role in recovery from blood stage malaria infection, and, also in modifying the immune system of the host. The phagocyte cells of liver (kupffer cells) also play an important role in host defence inflammatory process, immune mechanism and erythrocyte destruction. Apparently liver plays an important role in developing an immunity also.

However, very little is known about the role of high parasitaemia on pathological, biochemical and histochemical changes in the host liver following a course of malaria infection. For investigating the aforementioned aspect and other related issues, some of the steps found essential and the other details of the proposed study plans as they were carried out are as per below:

i) Maintenance of Plasmodium berghei.

ii) Albino mice, infected with P. berghei, were sacrificed at high parasitaemia. Their livers were excised and processed for, biochemical and histochemical studies by means of appropriate procedures (as described under materials and methods).

iii) Biochemical analysis of various constituents in liver included estimations for total lipid, total cholesterol, phospholipid, total carbohydrate, glycogen, glucose, total protein, total DNA and RNA contents.

iv) Determination of lipid peroxidation, GPT, GOT, acid and alkaline phosphatase contents.

v) Serum from infected albino mice was also collected to detect the extent of liver damage by estimating SGOT, SGPT, acid phosphatase, and alkaline phosphatase levels.

The immunization of albino mice against malaria was carried for checking the degree of immune protection, affordable.

The failure of conventional measures to control and eradicate malaria from tropical and subtropical areas of the world has ultimately led many scientists to search for a possible malaria vaccine. Therefore, the role of immunity, if any in the protection, or at least, in minimizing the patho-physiological changes and the accompanying alteration in biochemical parameters in malaria were studied in immunized P. berghei mice models as follows:

The detailed plan of study was as follows:

i) Antigen isolation: For the isolation of antigen, parasitized blood was collected in ACD. The leucocytes

were removed by alow speed centrifugation and by passing infected blood through a column packed with equal amounts of α -cellulose and microcrystalline cellulose.

ii) Parasitized RBC were lysed by saponin, the isolation of parasites was carried out on Histopaque. While parasites were disrupted by ultrasonication.

iii) Purity of P. berghei antigen was checked by ID, CIE and on PAGE (Poly Acryl Amide Gel Electrophoresis) and characterization of P. berghei antigen was carried out on sodium do-decyl polyacryl amide Gel electrophoresis (SDS-PAGE).

iv) Animals were immunized against P. berghei antigen using TDM as adjuvant. The details of the studies carried out were as follows:

a) Total leucocyte count

b) Serum enzyme estimations were done as follows:

1) Serum Glutamic Pyrvic Transaminase (SGPT).

2) Serum Glutamic Oxaloacetic Transaminase (SGOT).

3) Acid Phosphatase and Alkaline Phosphatase.

c) Humoral immune responses were studied in immune sera samples by using, ID, IHA and ELISA techniques.

d) The cell-mediated immune responses were assessed by employing delayed type skin hypersensitivity reactions and leucocyte migration inhibition tests.

e) Histological studies were carried out on the tissue sections obtained from liver, spleen and kidney of the infected, control and immunized animals.

v) The protective role, if any, of the elicited immune responses was assessed by challenging the immunized animals. The various parameters used to evaluate the immune protection of the host were:

a) Percent survival of animals.

b) Prepatent period.

c) Peak parasitaemia.

d) Average day of death.

e) Detection of the humoral and cell-mediated immune response, its magnitude and usefulness in host protection.

2.1 Animals

Healthy, male white rabbits, albino mice (Hissar Strain, India) were purchased from M/s Laboaids, Meerut.

2.2 Cell-Line

P. berghei (NK-65 strain) infected mice were obtained from Central Drug Research Institute (CDRI) Lucknow, India.

2.3 Anticoagulants

Acid citrate dextrose was obtained from Blood Bank, J.N. Medical College, Aligarh, while the other anticoagulant, sodium citrate was purchased from Sarabhai Merck Limited, India.

2.4 Gels

Agarose, Agar and Histopaque were purchased from Sigma Chemical Company, U.S.A.

2.5 Preservatives and Antibiotics

Sodium azide used as an antibacterial preservative was purchased from Riedal De-Haen-AG-Germany. Pinicillin-and streptomycin sulphide were purchased from Sigma Chemical Company, U.S.A.

2.6 Stains and Dye

Leishman stain and bromophenol blue were purchased from BDH, England, Coomassie blue (R-250) was purchased from Sigma Chemical Company, Methylene blue was purchased from Roanal, Hungary.

2.7 Conjugate and Substrate

Alkaline phosphatase and p-nitrophenyl phosphate were purchased from Sigma Chemical Company, U.S.A. Anti-mouse IgG (FITC) was purchased from Sigma Chemical Company, U.S.A.

2.8 Immunoadjuvant

Trehalose dimycolate (TDM) was supplied by Courtesy of (Late) Prof. Edgar Lederer, Laboratories de Biochemie, Central National de la Recherche Scientifique, 91190 Gif-sur-yvette France.

2.9 Chemicals

Analytical grade sodium phosphate (dibasic), potassium phosphate (monobasic), sodium chloride, sodium hydroxide, dextrose, sodium barbitone, sodium citrate, sodium carbonate, sodium bicarbonate, sorbitol, ferric chloride and

magnesium chloride were purchased from Sarabhai M. Chemicals, Company, India. Standard glycogen, DNA, RNA, bovine serum albumin obtained from Sigma Chemical Company, U.S.A. Sudan black, methyl green 1) pyronin, glucose oxide and peroxidase were purchased from Sigma Chemical Company, U.S.A.

The reagents purchased from S. Merck (India) were methanol, ethanol, glycerol, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium oxalate.

Hydrochloric acid, sulphuric acid, chloroform, acetic acid (glacial), iso-propanol, ammonium persulphate, perchloric acid, acetaldehyde, formaldehyde, N,N,N'; N'-tetramethylene-diamine (TEMED), N,N'-methylene bisacrylamide (Bis), glycine, sodium do-decyl sulphate, Tris-(hydroxy methyl)- aminomethane (Tris), sodium potassium tartarate, copper sulphate, sodium tetraborate, potassium hydrogen phthalate and barbituric acid were purchased from BDH (India).

All glass ware was sterilized before use and double glass distilled water was routinely used for making the solutions.

3.1.1 MAINTENANCE OF PLASMODIUM BERGHEI

The rodent malaria parasite, P. berghei (NK-65, strain), was maintained and allowed to multiply, in vivo, using albino mice as the animal model. About 2 x 10^6 P. berghei infected erythrocytes (in ACD) were inoculated in mice through intraperitoneal route. The size of the inoculum was 0.1 ml. On 8th day after inoculation, the mice were sacrificed and the blood collected directly from the heart in acid citrate dextrose. A small aliquots of the infected blood sample (.1 ml) was inoculated into fresh mice, while rest of the sample was pooled for antigen isolation. The cell line was therefore maintained by serial inoculation of parasitized erythrocytes. Saline injected mice were used as controls.

3.1.2 COUNTING OF PERCENT PARASITAEMIA

The inoculated animals were countinuously monitored for the presence of parasitized red cells through smears made from the tail tip of mice. The blood films were examined daily for estimating the levels of parasitaemia. These slides were stained with Leishman.

3.1.3 PREPARATION OF LEISHMAN STAIN

The Leishman stain solution was prepared by dipping 150 ml of Leishman stain powder wrapped in a small piece of

cotton in 100 ml of methanol for 24 hrs. at 37°C. The dried blood films were later allowed to further air dry. For staining the smears, equal volumes of Leishman stain and 0.01 M phosphate buffer, pH 7.2, were mixed and then layered on fixed, dried blood films. The slides were left for 30 minutes and then washed with 0.1 M phosphate buffer pH, 7.2.

Parasitized erythrocytes were examined and counted under microscope (Zeiss, Amplival) in oil immersion.

3.2 BIOCHEMICAL STUDIES

Body weight: Body weight of the infected animals was recorded at high parasitaemia.

i) Tissue Collection

Infected animals were scarificed under light ether anaesthesia and the liver tissues were excised and processed according to experimental design at 60-70% parasitaemia.

ii) Wet and Dry Weight of Liver

(a) **Wet Weight:** The whole liver was taken out from normal and infected animals at high parasitaemia, blotted dry on filtre paper and weighed quickly.

(b) **Dry Weight:** The liver tissues from the above animals were dried at 50°C for 24 hrs, weighed, dried again, washed till constant weight was obtained.

3.2.1 EXTRACTION OF LIPIDS

Liver (50-200 mg) was homogenized in a glass homogenizer to a final volume of 6 ml chloroform-methanol (2:1, V/V) according to the method of Folch et al (1951). Homogenate was shaken periodically for an hour and filtered through sintered glass funnel (G-4) under vacuum. The residue of each test tube was again homogenized with 2 ml chloroform-methanol and then filtered. The test tubes were rinsed with fresh chloroform-methanol and again filtered. The final volume of each extract was made upto 10 ml with fresh chloroform-methanol mixture. Thereafter 2.5 ml of normal saline solution was added to the extracts in each test tube (4:1 V/V). This was shaken vigorously on cyclomixer for complete mixing, and placed at -20°C in a deep freeze overnight for complete separation of the two layers. The junction of the two layers in each test tube was marked. The lower layer was collected in stoppered tubes with the help of a syringe and stored at -20°C until used. The test tubes in which the two layers separated were dried and the volume of lower layer in each test tube was measured. This extract was used for the estimation of total lipids, phospholipid and cholesterol.

3.2.2 ESTIMATION OF THE TOTAL LIPIDS

Total lipids were estimated according to the method of Woodman and Price (1972) described as follows:

i) Standard solution

A standard solution of 0.5 mg liver lipids/ml of choloroform (2:1) was prepared by diluting 1.0 ml refrigerated stock solution (50 mg liver/10 ml chloroform-methanol) in a 10 ml standard flask and made upto a volume of 10.0 ml with chloroform-methanol (2:1 V/V).

ii) Colouring Reagent

Six gram potassium dihydrogen orthophosphate and 0.3 g vanilline were dissolved by heating in a 100 ml volumetric flask and the volume was made up to 100.0 ml with double distilled water (DDW).

iii) Sulphuric Acid (Conc.) A.R. Grade

iv) Liver Lipids

Lipids were isolated from the mouse liver by the technique described earlier (Folch et al.,1951). The extract was dried by vacuum rotatory evaporator at 40°C. The dried lipid preparations were stored at 4°C.

v) Procedure

An 0.1 ml of duplicate liver extract and a duplicate set of standard with 50 to 500 ug of liver lipids were taken in 18 x 150 mm Corning test tubes. Each tube after receiving 2.5 ml conc. sulphuric acid was heated in boiling water bath for 20 min. After cooling, 5.0 ml colouring reagent was added and absorption was read at 530 nm after 10 min. against a reagent blank by Beckman DU-6 spectrophotometer. Calculations were made as under:

$$\text{Total Lipids (mg/g fresh weight)} = \frac{C \times V}{Vt \times Wt}$$

where, C = Concentration of lipids in ug/0.1 ml liver extract.

V = Total volume of lower layer

Vt = Volume taken for estimation

Wt = Fresh weight of the tissue in mg.

The above formula was also used for calculating the concentration of phospholipid and cholesterol.

3.2.3 ESTIMATION OF PHOSPHOLIPIDS

Phospholipid was estimated by the method of Fiske and Subbarow, as described by Marinetti (1962).

i) A standard solution of 0.01 mg inorganic phosphate/ml was repared by diluting 5.0 ml refrigerated stock solution (0.438 g KH_2PO_4/500 ml DDW) in a 100 ml standard flask with DDW.

ii) Perchloric acid 70% A.R.

iii) Ammonium molybdate solution 2.5%.

iv) Reducing Reagent

Reducing reagent was prepared by dissolving, 3.0 g sodium bisulphite, 0.6 g sodium sulphite and 0.05 g recrystallized, 1-amino-2-napthol-4-sulphonic acid (ANSA) in 25 ml DDW. A slight yellow solution thus obtained was stored in amber coloured bottle. The colour was found stable for about a week at room temperature.

v) Recrystallization of ANSA

Fifteen gram sodium metabisulphite, 1.0 g sodium sulphite (anhydrous) and 1.5 g crude ANSA were dissolved in 100 ml DDW by heating on boiling water bath. Hot solution was filtered through the filter paper. One millilitre conc. HCl was added in the filtrate and stirred. Precipitate was filtered with suction pump, washed in about 30 ml double distilled water and finally with alcohol till washing was colourless. This purified ANSA was dried in an oven at 100°C for 1 hour with least

possible exposure to light and was transferred to an amber coloured bottle.

vi) Procedure

An 0.2 ml of duplicate liver lipid extract (lower layer) was placed in 18 x 150 mm corning test tubes and all the solvent was dried up by heating in a boiling water bath. Then 1.0 mililitre AR grade 70% perchloric acid was added to the samples, after which they were heated on a digester for 30 min. or until the sample became clear. After complete digestion, samples were cooled at room temperature. There after, 1.5 ml ammonium molybdate, 0.2 ml reducing reagent and 7.0 ml DDW were added with vigorous shaking after each addition. The test tubes were heated again in a boiling water bath for 7 min. After 30 min, the colour intensity was read at 700 nm. A calibration curve was plotted using 1 ug to 8 ug of phosphorous and 1.0 ml perchloric acid. A blank was prepared with 1.0 ml perchloric acid alone.

vii) Calculation

The absorption of light being a linear function of the phosphorus content in a solution, its concentration in an unknown sample can be calculated as directly proportional to the absorbance obtained from the standard solution. The amount of phospholipid was calculated by multiplying with a factor of 25.

3.2.4 ESTIMATION OF CHOLESTEROL

Cholesterol was estimated by Lieberman-Butchard reaction, as described by Bloor et al. (1922).

Reagents

i) Standard

Cholesterol standard at a concentration of 1.0 mg/ml in chloroform was prepared by diluting 1.0 ml refrigerated stock solution of recrystallized cholesterol (100 mg/10 ml chloroform) in a 10 ml standard flask and the volume made upto 10 ml by the addition of AR grade chloroform.

ii) Colouring Reagent

50 ml of AR grade acetic anhydride was first placed in a deep freeze for 1 hour, and then 5.0 ml conc. sulphuric acid (AR) was added to it drop-wise with constant stirring. It was allowed to stand for an additional 10 min. before using. This reagent was prepared fresh for each use.

iii) Procedure

An 0.5 ml of liver lipid extract was taken in 15 x 125 mm screw capped culture tubes. To this, 4.5 ml chloroform (AR) and 1.0 ml colouring reagent were added and

mixed well. The tubes were kept in dark at 25°C for 30 min. The absorbance was read at 660 nm against a reagent blank. A calibration curve with different concentrations of cholesterol (100-800 ug) was drawn according to the same procedure as described above.

3.2.5 ESTIMATION OF LIPID PEROXIDATION

The amount of malonaldialdehyde formed/30 min. of lipid peroxidation was estimated according to the procedure of Utley et al. (1967), as described below:

Reagents

i) **0.15 M Potassium Chloride**

2.2368 g KCl dissolved in 200 ml DDW.

ii) **10% (W/V) Trichloroacetic Acid**

10 g TCA dissolved in 100 ml DDW.

iii) **0.67% 2-Thiobarbituric Acid (TBA)**

This was prepared by dissolving 0.67 g TBA in 25-50 ml DDW by adding two pellets of NaOH. The pH of the solution was adjusted to 7.2 with the help of 1N HCl. The final volume was made upto 100 ml with DDW.

iv) Procedure

The liver was homogenized (10%, W/V) in chilled 0.15 M KCl. One ml of each homogenate was taken in a 25 ml conical flask and incubated at 37°±1°C in a metabolic shaker (120 strokes/min; amplitude 1 cm) for 2 hrs. After incubation, 1.0 ml of 10% TCA was added to each sample, both the incubated as well as non-incubated samples were contrifuged at 3000 rpm for 10 min. One ml of the clear supernatant was mixed with 1.0 ml of 0.67% TBA and 1.0 ml DDW and the tubes were placed in a boiling water bath for 10 min. cooled and the absorbance of the colour was measured at 535 nm.

vi) Calculation

Lipid peroxidation was calculated using the following formula:

$$X = \frac{OD \times 30 \times 1000 \times 1000 \times 1000 \times 3 \times 2}{1.56 \times 100{,}000 \times 1000 \times 180}$$

or $$X = \frac{OD \times 10}{1.56}$$

where, X = Nanomoles of malonaldialdehyde formed/30 min.

OD= Change of optical density at zero hour and 2 hour incubation of the same sample.

3.2.6 ISOLATION OF NUCLEIC ACIDS

Nucleic acids were isolated following the method of Searchy and MacInnis (1970). Weighed liver tissues were homogenized in 5.0 ml of 0.5 N perchloric acid. The homogenates were heated at 90°C in boiling water bath for 10 min then cooled and centrifuged at 3000 rpm for 10 min. Supernatants were taken in the graduated test tubes and the volume was maintained upto 5 ml with 0.5 N perchloric acid. This extract was used in the estimation of DNA and RNA.

(A) ESTIMATION OF DEOXYRIBONUCLEIC ACID

DNA was estimated following the method of Burton (1956).

Reagents

i) Diphenylamine Reagents

One and a half gram diphenylamine was dissolved in about 50-60 ml conc H_2SO_4 and the final volume was brought to 100 ml.

ii) Procedure

Two ml supernatant of the nucleic acid extract was taken in a test tube. To this, 4.0 ml diphenylamine reagent

was added, and the tubes were heated on boiling water bath for 15 min. After cooling, the colour intensity was measured at 600 nm against a reagent blank.

(B). ESTIMATION OF RIBONUCLEIC ACID

RNA was estimated by the method of Dische (1955).

i) Orcinol Reagent

Thirty three mg ferric chloride was dissolved in about 50 ml conc. HCl and 3.5 ml of 6% orcinol (dissolved in absolute alcohol) was mixed in it, and the volume was made upto 100 ml with HCl.

ii) Procedure

Two ml supernatant samples of the above were taken in the tubes to which 4.0 ml of the orcinol reagent was added. Test tubes were heated on a boiling water bath for 15 min then cooled and the absorbance read at 660 nm against a reagent blank.

iii) Calculation

Both DNA and RNA were calculated by the following formula.

DNA and RNA (mg/g fresh tissue wt)

$$= \frac{C \times V}{Vt \times Wt}$$

where, C = Concentration in ug in 2.0 ml extract

Vt = Volume taken for the estimation

V = Total volume of the extract (4 ml)

Wt = Fresh weight of the tissue in milligram

3.2.7 ESTIMATION OF PROTEIN

Protein was analysed following the well known method of Lowry et al. (1951).

Reagents

i) A standard solution of 1.0 mg BSA/ml was prepared in PBS.

ii) Copper Reagent

Reagent A : 4% Sodium carbonate in DDW

Reagent B : 2% Copper sulphate in DDW

Reagent C : 4% Sodium-potassium tartarate in DDW.

Copper reagent was prepared by mixing reagents A, B and C in a ratio of 100 : 1 : 1 respectively at the time of use.

iii) Folin-Ciocalteu-Reagent

iv) Procedure

Residue left in the test tubes after taking the supernatant for nucleic acid estimation was dissolved in 5.0 ml DDW. An 0.1 ml aliquot was taken in the test tubes from this solution and the volume was brought upto 1.0 ml with DDW. To this, 5.0 ml of copper reagent was added and the tubes were shaken thoroughly on a cyclo-mixer. After 10 min 1.0 ml Folin's reagent was added. The colour intensity was measured at 700 nm after 30 min against a reagent blank.

3.2.8 ESTIMATION OF GLYCOGEN, CARBOHYDRATES AND GLUCOSE

(A) Glycogen Estimation

Glycogen was extracted according to the method of Le Baron (1955) and estimated colorimetrically as described by Montogomery (1957).

Reagents

i) Standard Solution

Glycogen standard of 0.1 mg/ml in DDW was prepared by dissolving 5 mg glycogen in 50 ml of DDW.

ii) TCA (5%)

5 gm TCA was dissolved in 100 ml DDW.

iii) Phenol (80%)

Eighty gm phenol was dissolved in 100 ml DDW.

iv) Ethanol (96%)

v) Conc. Sulphuric Acid

vi) Procedure

a) Extraction

A known quantity of tissue was homogenized in 5 ml of 5% freshly prepared TCA and centrifuged at 3,000 rpm for 10 min. The supernatant was taken and made upto a concentration of 70% with 96% ethanol. It was centrifuged again at 3,000 rpm for 10 min. The supernatant was removed and the residue dissolved in DDW. It was then transferred to 5 ml volumetric flask and finally made upto 5 ml with DDW.

b) Estimation

A 2 ml aliquot was taken in each test tube and then 0.2 ml of 80% phenol and 5 ml of conc. sulphuric acid was added. Tubes were shaken thoroughly and allowed to stand for 30 min, at room temperature. The colour intensity was read

at 490 nm. A calibration curve with different concentration of glycogen (10-60 ug) was drawn according to the same procedure as described above. The values were plotted by the least square method and expressed as mg/g tissue.

(B) Total Carbohydrate

Acid hydrolysis of the liver homogenate was carried out for the estimation of carbohydrate and glucose.

For acid hydrolysis equal volume of 1.5 N HCl were used to hydrolyze equal aliquots of normal and infected liver homogenates. The hydrolysis reaction was performed at 110°C for 10-12 hrs inclosed containers. It was cooled and filtered. The filtrate was neutralized with 0.1 NaOH.

The amount of total carbohydrate was estimatated according to the method of Montogomery (1957).

(C) Glucose Estimation

Total glucose was estimated according to the method of Bergmeyer and Bernut (1963).

Reagent

i) Standard Solution

Glucose standard of 0.1 mg/1 ml in DDW was prepared by dissolving 5 mg glucose in 50 ml of DDW.

ii) O-Tolidine (1%)

This was prepared by dissolving 1 gm O-Tolidine in absolute ethanol by using magnetic stirrer.

iii) Sodium Acetate Buffer (pH 5)

This was prepared by mixing approx. 3. Volume of 0.15 M acetic acid to 7 volume of 0.15 Na acetate. The pH of the this solution was adjusted at pH 5.

iv) Peroxides

This was prepared by dissolving 20 mg peroxidase in 100 ml acetate buffer.

v) Glucose Oxidase Reagent

Glucose oxidase reagent was prepared by dissolving 200 mg of glucose oxidase to 80 ml acetate buffer and 1 ml of O-tolidine solution. The solution was make upto 100 ml with buffer.

vi) Procedure

The final preparation of homogenate for glucose estimation was carried out same as used for glycogen and carbohydrate.

vii) Estimation of Glucose

A 2 ml of aliquot was taken in tubes, then 3 ml of glucose oxidase reagent was added in each tube at 2 minutes interval. The solution was mixed gently and set aside for exactly 10 minutes. When blue coloured appeared in tubes, the optical density of solution was measured at 480 nm.

3.2.9 ENZYMATIC ASSAYS IN LIVER TISSUES

Livers of infected mice were excised at high parasitaemia and washed in cold KCl (150 mM), and homogenized in same medium to give a 10% (W/V) homogenate. Homogenization required 60-120s with a motor driven Teflon pestle. The suspension was centrifuged at 15,000 x g for 20 min. and the supernatant solution was transferred to clean tubes. Freezing and thawing of the supernatant was done a number of times to release acid and alkaline phosphatase before assays.

(A) Estimation of Liver Transaminases

Aliquots from suitably diluted liver homogenate were taken separatly for glutamic pyruvate transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) determination. It was calculated according to the method of Reitman and Frankel (1957) using sodium pyrvate as a standard.

Preparation of Reagents

i) Phosphate buffer (0.1 M, pH 7.4)

A 420 ml volume of 0.1 M disodium phosphate was mixed with 80 ml of 0.1 M potassium dihydrogen phosphate.

ii) Pyruvate 2 mM per litre (for standard curve)

A 22.0 mg sample of sodium pyruvate was dissolved in 100 ml of phosphate buffer.

iii) α-Ketoglutarate, 2 mM per liter, dl-alanine, 200 mM per liter (for GPT substrate)

About 29.2 mg of α-Ketoglutaric acid was placed with 1.78 Gm dl-alanine in a small beaker. The pH was adjusted at 7.4 with sodium hydroxide and the solution transferred quantitatively with buffer solution to a 100 ml volumetric flask, and then diluted to the mark with buffer solution.

iv) α-Ketoglutarate, 2 mM per litre, diasparate, 200 mM per liter (for GOT substrate and standard curve)

A 29.2 mg sample of α-Ketoglutaric acid was placed in 2.66 Gm. dl aspartic acid in a small beaker. The above preparations were adjusted to a pH of 7.4 with sodium hydroxide and transferred quantitatively with buffer to a 100 ml volumetric flask, and then diluted to the mark with buffer solution.

v) 2,4-dinitrophenylhydrazine, 1mM per liter

About 19.8 mg of 2,4-dinitrophenylhydrazine was dissolved in 100 ml of 1N hydrochloric acid.

vi) Sodium Hydroxide, 0.4 N

vii) Procedure

One millilitre of the desired substrate was pipetted into a test tube, and placed in a water bath at constant temperature (40°C) for 10 min. Upon the addition of 0.2 ml of homogenate the contents were mixed, and after an incubation period of exactly 30 min. for GPT or 60 min. for GOT, the tubes were removed from the water-bath. One millilitre of the 2,4-dinitrophenylhydrazine reagent was added immediately, thereby stopping the reaction. After the tubes were allowed to stand at room temperature for a minimum of 20 min.,10 ml of 0.4 N sodium hydroxide was added. A rubber stopper was inserted to each tube while the contents were mixed by inversion. At the end of exactly 30 min. the optical density of the solution was measured at 505 nm, using water as the blank.

While the specimens were incubating, a control tube for each homogenate sample was prepared. One ml of the substrate, 0.2 ml of homogenate and 1 ml of 2,4-dinitrophenyl-hydrazine reagent were mixed in a test tube. After 20 min.

10 ml of 0.4 N sodium hydroxide was added and optical density of the solution was measured.

The level of enzyme activity was calculated in terms of micromoles of the keto acid formed per min. per gram tissue protein.

(B) Estimation of Liver Phosphatases

(a) Acid Phosphatse

Acid phosphatase activity was determined according to a slightly modified method of Nelson (1966). A typical reaction mixture in a final volume of 2.0 ml contained 0.5 ml homogenate, 6.0 u moles of ethylenediaaminetetraacetic acid (EDTA), 75 u moles of p-nitrophenyl phosphate (disodium salt) and 150 u moles of acetate buffer (pH 5.0).

(b) Alkaline Phosphatase

The enzyme conc. of alkaline phosphatase in liver was measured according to the method of Morton (1954). For the estimation of conc. of alkaline phosphatase, the reaction mixture in a total volume of 2.0 ml contained, 75 u moles of p-nitrophenyl phosphate (disodium salt), 3 u moles of $MgCl_2$, 150 u moles of bicarbonate buffer (pH 9.5) and 0.5 ml of a suitably diluted enzyme.

Procedure

Test tubes containing the reaction mixtures were incubated for 30 min. at 37°C with occassional shaking. The reaction was stopped by addition of 2.0 ml NaOH (.1 N). The enzymes in control tubes were supplemented after stopping the reaction. One Unit of enzyme was that amount which liberated 1 u moles of p-nitrophenol/min.

3.2.10 ENZYMATIC ASSAYS IN SERUM

Preparation of Serum

The blood from normal, infected, immunized animals was collected in clean and dry tubes. It was kept at room temperature, for 30-60 minutes, then, at 37°C for 30 minutes. Finally, it was kept at 4°C overnight. Next day it was centrifuged at 4000 rpm for 20 minutes. The clear serum (supernatant) was transferred to small vials and stored at - 20°C.

(A) Estimation of Serum Transaminases

Serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) in normal, and infected serum was estimated according to the procedure of Reitman and Frankel (1957) using sodium pyruvate as a standard.

The level of enzyme activity was calculated in terms of micromoles of pyruvate formed per min, per ml of serum.

(B) Estimation of Serum Phosphatases

The activity of acid and alkaline phosphatase was determined by a modification of the procedure described by Nelson (1966) and Morton (1954). Rest of the procedure was the same as used for liver phosphatase estimations.

Statistical Analysis

The data were analyzed using student's 't' test. Significant differences between means of experimental and control groups were calculated and 'p' values less than 0.01 were considered as significant.

3.2.11 HISTOCHEMICAL METHODS

Slice of liver tissues (2-3 u thick) from normal and P. berghei infected animals were collected in chilled normal saline. After a brief wash in saline, the tissue pieces were kept in 5% polyvinyl alcohol solution for 15 min. and mounted on microtome chucks over a drop of water and "quenched" (Snapfrozen) in liquid nitrogen at about -190°C. The tissue were then transferred to a microtome chamber (Slee, England), maintained at -25°C. About 7-8 um thick sections were cut

and mounted on clear glass slides. The sections were air dried and used for different histochemical reactions immediately.

(A) Histochemical Staining of Total Lipids

Staining of total lipid was carried out according to the method of Lisen and Dagnelic (1955).

Reagent

Soln A: Sudan black - In 1% Isopropanol

Soln B: Na - Borate 1% in DDW.

Soln C: Soln A & B were mixed in equal parts and allowed to stand for 10 min. at room temp and then filtered.

(i) Formal Calcium Chloride Solution

One gram $CaCl_2$, was dissolved in about 50 ml DDW. to this solution, 10 ml formaldehyde (40%) was added and the volume was made upto 100 ml with DDW.

(ii) Glycerine Jelly

Gelatin (50 gm) was dissolved in 100 ml DDW with moderate heating (60°C) and then 100 ml glycerol was added while the solution was mixed well. The solution was then filtered. The bottle was stored in a refrigerator.

iii) Procedure

Formal ca-fixed frozen sections (lou in thickness) were cut on a freezing microtome. The sections were mounted on glass slides and air dried at room temperature. These slides were washed with DDW and stained for 10 mts. in solution C. These were differentiated in 60% isopropanol and washed with DDW. The sections were counterstained with the red nuclear dye (carmalum, 0.1%), and mounted in glycerine jelly after washing the sections. The lipid material, was stained black and the nuclei red.

(B) Histochemical Localization of Nucleic Acid

Methyl green pyronin method (Kurnick, 1950) was used for localization of DNA and RNA.

Pyronin Y = 2 gms. dissolved in 100 ml DDW. The extraction of pyronin was carried out by chloroform.

Methyl green: 2 gm methyl green was dissolved in 100 ml DDW. The extraction of methyl green was carried out in chloroform.

The slides were stained in the solution for 6 min. where DNA was the stained black and the RNA bright red.

3.3 IMMUNOLOGICAL STUDIES

3.3.1 ANTIGEN PREPARATION

Antigen preparation was carried out in several steps detailed below:

(A) Isolation of Parasitized Erythrocytes

Removal of leucocytes, buffy coat and platelets from the infected blood was carried out by the following methods.

(a) The infected blood was washed several times with chilled normal saline

When parasitemia was about 60-80% on 8th day after inoculation, the blood was collected directly from the heart in acid citrate dextrose (anticoagulant). The pooled cells were washed by centrifugation in chilled normal saline (pH 7.2) at 1500 rpm for 20 min. at 4°C. The buffy coat containing leucocytes and plasma platelets were discarded and the sediment essentially consisting of normal and parasitized erythrocytes pooled. The pooled blood was further washed four times by centrifugation at 1500 rpm for 20 min. at 4°C.

(b) Leucocyte removal by passing the whole infected blood through a column

Leucocytes were further removed by passing whole blood through a column packed with ℂ-cellulose and

microcrystalline cellulose (mean size; 50 microns; Sigma cell 50) (Beutler et al., 1976). This was done within 7 days after the collection of blood.

i) Swelling of the Cellulose

Equal amounts (by weight) of ⍺-cellulose and micro-crystalline cellulose were suspended in excess of 0.154 M NaCl. This suspension was kept overnight at room temperature.

ii) Packing the Column

The column was made in a 5 ml sterile plastic syringe. The syringe was clamped in a vertical position with the help of stand. Inner diameter of the syringe was 1.26 cm. The slurry was poured in the column with a glass rod. When cellulose particles were settled, a pressure was applied for homogeneous packing. A column of 2 ml was found quite adequate.

iii) Loading and Elution of the Column

One ml of acid citrate dextorse treated blood was applied on a 2 ml column and eluted with 2 ml of saline. The flow rate was maintained at 18 ml/hr.

(B) Lysis of Parasitized Erythrocytes

After removal of leucocytes from infected blood, the washed erythrocytes were collected and subjected to saponin

induced lysis (30 minutes) in equal volumes of 0.2% saponin in normal saline with continuous stirring in an ice bath. The lysate was centrifuged at 4000 rpm for 1 hour at 4°C. The supernatant was discarded, while the pellet was again washed 3-4 times with chilled normal saline for removing the erythrocyte debris. After final washing, the pellet containing malaria parasites was suspended in chilled 0.25 M sucrose solution.

(C) Purification of Parasite

This was carried out on Histopaque (Sigma Chemical Company). Histopaque was used as an aqueous solution having a density of 1.077±0.001. It contained 5.7 gm of Ficoll 400 and 9 gm of diatrizoate sodium per 100 ml. Histopaque was dispended in a centrifuge tube. An equal volume of the impure parasite suspension was layered on it. This was centrifuged at 400 g for 30 minutes at 4°C in a Remi K-25 model centrifuge. The released parasites appearing in a brown band, at the interface were withdrawn with the help of a pasteure pipette. The isolated parasites were washed thrice and resuspended in normal saline (0.154 M NaCl) at a concentration of 2×10^9 parasite/1 ml. The above antigen preparations was stored at -20°C. The purity of parasite obtained after density gradient centrifugation was checked microscopically. The parasite smears were dyed with Leishman stain. The examination procedure was

same as used for blood smears. The slides were examined under an oil immersion objective of the microscope.

(D) Preparation of Soluble Antigen

The parasite suspension was subjected to ultrasonication for the disruption of parasites. The ultrasonication was performed in 3 pulses, for a total of 6 minutes in a 50 Watt. 9 KC Magnetostriction oscillator. The suspension thus obtained was centrifuged at 10,000 rpm for 1 hr. at 4°C. The supernatant was collected and used as soluble antigen.

3.3.2 CHECKING THE PURITY OF ISOLATED ANTIGEN

The purity of parasite antigen preparation was checked immunologically and by electrophoretic methods. In immunological tests, the antigen samples containing disrupted parasites were checked against normal mouse RBC antiserum.

(a) Production of Normal Mouse RBC Antibodies

Blood was collected from normal healthy mice in ACD used as an anticoagulant. Washing of the blood sample was done three times with chilled normal saline (pH 7.2), and the washed RBC's were subjected to extrusion in a 10% suspension of erythrocytes by passing it through a syringe fitted with a 27 gauge needle (Chow and Kreier, 1972).

(i) Immunization of Rabbits Against Mouse RBC

The mouse RBC samples were inoculated into rabbits through intravenous injections.

Male healthy rabbits were immunized against whole mouse RBC antigen preparation. The first four injections containing 0.2 ml of the above antigen were given intravenously on day 1st, 2nd, 3rd and 4th. The 5th, 6th and 7th injections consisting of a similar dose were given on day 6th, 8th and 10th. After a rest period of two days, the 8th, 9th and 10th injections of 0.3 ml were given intravenously on days 13, 15 and 17. The blood was drawn at weekly intervals from heart, for checking antibody activity and the sequential appearence of immunoglobulins in weekly sera samples. The immunization schedule followed during the course of these investigations is given in Table 1.

(ii) Collection of Antiserum

After completing the entire course of immunization, small samples of blood were initially withdrawn for detecting antibody activity. Animals showing a good antibody response were used for obtaining blood from the heart.

(b) Antibody Detection

The IHA test was performed for the detection of

antibodies in serum samples obtained from immunized rabbits against NME (normal mouse erythrocyte extract).

Indirect Haemagglutination Test

The indirect haemagglutination (IHA) test was performed according to the method of Mathews et al. (1975) with some modifications.

Sheep 'O' erythrocytes (ORBC) were washed 4 times with isotonic buffered saline, pH 7.2. After final washing, a 3% (V/V) suspension of ORBC was prepared in isotonic phosphate buffer saline, pH 7.2. This suspension was mixed with an equal volume of 1/20,000 tannic acid and incubated at 4°C for 30 minutes with intermittent shaking. Tanned ORBC were collected by centrifugation and washed three times with phosphate buffered saline, pH 7.2, containing 0.5% bovin serum albumin to achieve a final concentration of 3% (V/V). A 3% suspension of tanned ORBC was mixed with an equal volume of antigen at appropriate dilution and incubated at 37°C for 30 minutes with intermittent shaking for antigen coating. The antigen coated ORBC were collected by centrifugation and then washed 3-4 times with phosphate buffered saline, pH 7.2, containing 0.5% bovin serum albumin. Finally, a 1.5% (V/V) suspension of antigen coated ORBC was prepared for use in the tests.

TABLE I

Immunization Schedule in Rabbits

Groups	No. of animals	Immunization	Immunization doses (ml) (days of inoculation)									
			1st	2nd	3rd	4th	6th	8th	10th	13th	15th	17th
I	4	Control Animals Saline (m)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.3
II	4	Experimental Animals (Mouse RBC Antigen/ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.3

To each well of the 'U' bottomed microtitre plate, an 0.025 ml sample of serially diluted antiserum was added alongwith an equal volume of antigen coated ORBC suspension. The plates were sealed with transparent gummed tape and gently shaken for 5 minutes at room temperature. The plates were incubated at room temperature in humid chambers for 1 hour and then kept over night at 4°C. The highest dilution of the test serum giving a positive carpet like pattern was recorded as the titration end point.

This test was repeated weekly for detecting the antinormal mouse RBC antibodies raised in rabbit against normal mouse erythrocytes.

(A) Immunodiffusion

This test was performed according to the published procedure of Quchterlony (1949). Agarose was dissolved in 0.05 M veronal buffer, pH 8.6, containing 0.02% sodium azide. The concentration of agarose was 0.8%. The melted agarose was poured on glass slides to form approximately 1.5 mm thick layer of uniform thickness. The slides were left undisturbed, allowing the gel to solidify. The wells were punched in the solidified gel.

The central and peripheral wells were filled with antiserum and antigen at appropriate dilutions. The test slids

were kept for 4 hours at room temperature in a moist chamber and subsequently at 4°C for 72 hours. The precipitin bands were visible either before staining, or after staining with Coomassie brilliant blue.

(B) Counter Immuno Electrophoresis

This test was performed according to the method of Bjerrum and Bog Hansen (1976). The method of slide preparation for CIE was the same as for immunodiffusion. On agarose coated slides, a set of wells was cut with a 2 mm punch. The centre to centre distance between the well was 5 mm.

The cathodal wells were filled with the antigen, while the anodal wells were loaded with antiserum. Electrophoresis was performed for about 40-50 minutes. The slides were then incubated over night at 4°C in a moist chamber. Photographs were taken either before or after staining with Coomassie brilliant blue.

(C) Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamine gel electrophoresis was carried out for checking the purity of isolated antigen preparations against RBC contaminant. This was performed according to the method of Laemmli (1970).

Preparation of Reagents

(a) Stock solution

The stock solution employed in polyacrylamide gel electrophoresis was filtered and stored at 4°C in brown glass bottles. The stock solutions were as follows:

i) Reservoir buffer

Three gms of tris - (hydroxymethyl) - aminomethan and 14.40 gm of glycine were dissolved in 1 litre of distilled water and the pH adjusted at 8.3 with 1N HCl.

ii) Acrylamide solution

Thirty gms of acrylamide and 0.8 gm of recrystallized N,N′-methylene bisacrylamide were dissolved in 100 ml distilled water.

iii) Lower Tris

One and half M tris - (hydroxymethyl)-aminomethane solution was prepared in distilled water and the pH adjusted at 8.8 with 1N HCl.

iv) Upper Tris

One and half M tris - (hydroxymethyl) -

aminomethane was made in distilled water and the pH adjusted at 6.8 with IN HCl.

v) Sample Buffer

Ten percent sucrose solution was prepared in upper tris. This also contained 0.022% bromophenol blue.

vi) Staining Solution

About 0.1 percent Coomassie blue (R-250) was dissolved in 25 percent methanol and 10 percent acetic acid in water (V/V/V). It was filtered immediately before use.

vii) Destaining Solution

A 7 percent acetic acid (glacial) and 25 percent methanol was prepared in 1 litre DDW water (V/V/V).

(b) Working solutions

These solutions were prepared on the day they were used.

(i) Ammonium Persulphate Solution

A 10 percent ammonium persulphate was prepared in distilled water.

ii) Lower Gel Solution (7%)

A 7 percent polyacrylamide gel was prepared immediately before use. The solution was prepared as follows:

Distilled water	20.4 ml
Lower tris HCl (4 times diluted)	10.0 ml
Acrylamide solution	9.38 ml
Ammonium persulphate solution	0.28 ml
N,N,N',N'-tetramethyl-ethylenediamine (TEMED)	15 λ

iii) Upper Gel Solution

This solution was also prepared immediately before adding to the gel tubes. It contained:

Distilled water	3.25 ml
Upper tris HCl (4 times dilluted)	1.25 ml
Acrylamide solution (30%)	0.50 ml
Ammonium persulphate solution	15 λ
N,N,N',N'-tetramethyl-ethylenediamine(TEMED)	15 λ

iv) Preparation of Sample

The antigen samples were adjusted to a concentration of about 1 mg protein/ml in the sample buffer.

(c) Procedure

The clean and dimethyldichlorosilane treated PAGE tubes (10 x 0.6 cm) were positioned vertically. A 7 percent lower gel solution was transferred to the tubes upto a 8 cm height, on top of which water was layered in such a way that gel was not disturbed. The tubes were allowed to stand for 40 min. for polymerization. Then the water layer was removed and the upper gel solution layered upto a height of 1 cm. The water was again layered on top of it and the tubes left undisturbed for 60 min. These tubes were held in their respective positions in electrophoresis chambers. A 25 ul sample was loaded into each gel tube. The test was run at 100 volts at 4 mA current per gel tube for 8 hrs.

(d) Staining and Destaining of the Gel

The gel was stained for 16 hrs. in the staining solution, and then destained in a 7% acetic acid solution containing 25 percent methanol.

3.3.3 BIOMOLECULE ESTIMATIONS

(A) Protein Estimation

Protein concentration was estimated by Lowry's method (Lowry et al., 1951), as described before.

The calibration curve was plotted using standard solution of bovine serum albumin of 100 mg/100 ml strength. About 5 ml fresh prepared copper reagent was added to the protein sample. After incubating for 10 minutes at room temperature. 1 ml of 1:4 (V/V) diluted Folin's reagent was added. Following 30 minutes incubation in dark, the colour intensity was measured at 700 nm wave length, by using a Bausch and Lomb spectronic-21 spectrophotometer.

(B) RNA Estimation

RNA concentration was determined by Orcinol-reaction Sanlin and Schjeide, 1969).

Reagents

i) Orcinol Reagent

Orcinol reagent was prepared by adding ferric chloride (0.5 percent) in 1 percent hydrochloric acid.

ii) Procedure

Equal volumes of RNA sample and Orcinol reagent were mixed and kept in a boiling water bath for 20 min. The tubes were cooled and the absorbance was recorded at 660 nm.

(C) DNA Estimation

Burton's method (Burton, 1956) was used for the estimation of DNA.

i) Diphenylamine Reagent

This was prepared by the addition of 750 mg of diphenylamine to 50 ml of glacial acetic acid containing 0.75 ml concentrated H_2SO_4.

ii) Procedure

About 1 ml sample solution was mixed with 1 ml of 1 N perchloric acid. The tubes were kept at 70°C in a water bath for 15 minutes. Then 0.1 ml of 5.43 mM acetaldehyde was added, followed by 2 ml of diphenylamine reagent. The tubes were allowed to stand at room temperature for 16 hrs. The absorbance was recorded at 600 nm.

(D) Hexoses Estimation

Winzler's method was used for estimation of hexoses (Winzler, 1955).

i) Orcinol Sulphuric Acid Mixture

Orcinol sulphuric acid mixture was prepared by the addition of orcinol (1.6 percent in distilled water) and 60 percent sulphuric acid in a ratio of 1:7.5 respectively.

ii) Procedure

A sample in 1 ml portion was mixed with 8.5 ml orcinol sulphuric acid mixture. The tubes were kept at 80°C

in a water bath for 15 min. After cooling the tubes, absorbance was recorded at 505 nm.

3.3.4 CHARACTERIZATION OF P. BERGHEI ANTIGEN BY SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was carried out by the method of Laemmli (1970) with slight modifications.

a) Stock Solution

The stock solutions were prepared and stored at 4°C in brown glass bottles.

i) Stacking Gel Buffer or Upper Gel Buffer

0.5 M Tris - (hydroxymethyl) - aminomethane containing 0.4 percent SDS-Lauryl sulphate were dissolved in 1 litre distilled water. The pH of this solution was adjusted at 6.8 with 1N HCl.

ii) Separating Gel Buffer or Lower Gel Buffer

This was prepared by dissolving 1.5 M Tris-(hydroxymethyl) aminomethane and 0.4 percent SDS-Lauryl sulphate in 1 litre distilled water at 8.8 pH.

iii) Electrophoresis Buffer

A solution containing 0.05 M Tris-(hydroxymethyl)-

aminomethane, 0.384 glycine and 0.1 percent SDS Lauryl sulphate was prepared in 1 litre distilled water. The pH of this solution was adjusted at 8.3 by using 1N HCl.

iv) Acrylamide Stock Solution

Samples of 30 percent acrylamide and 0.8 percent N,N'-methylene bisacrylamide were dissolved in distilled water.

v) Gel Fixer

The gel fixer used was 25 percent isopropanol and 10 percent glacial acetic acid in distilled water (V/V/V).

vi) Staining Buffer

About 0.2 percent Coomassie blue (R-250) was dissolved in 25 percent methanol and 10 percent acetic acid (glacial) in distilled water (V/V/V).

vii) Destainer

The destainer used in this study contained 25 percent methanol and 10 percent acetic acid made in distilled water (V/V/V).

(b) Working Solutions

These solutions were prepared immediately before use.

i) Ammonium Persulphate Solution

A 10 percent ammonium persulphate solution was freshly prepared in distilled water.

ii) Composition of Separating Gel (10%)

Acryl amide solution	13.33 ml
Resolving gel buffer	5.00 ml
Double distilled water	19.266ml
Ammonium persulphate	2 ml
TEMED	0.02 ml

iii) Composition of Stacking Gel

Acryl amide solution	1.3 ml
Stacking gel buffer	2.6 ml
Double distilled water	6.026 ml
Ammonium persulphate	0.53 ml
TEMED	0.01 ml

iv) Composition of Solublizing Buffer

Sample solublizing buffer	1.85 ml
B-mercaptoethanol	0.05 ml
1% W/V Bromophenol blue	0.1 ml

(c) Procedure

Thoroughly clean and dimethyldichlorosilane treated glass tubes were placed in racks in an exactly vertical position. The components of the separating gel were thoroughly mixed. The gel solution was immediately dispensed in tubes; and overlayed with water. The tubes were left undisturbed for about 30-40 min and then the water layer was removed. After this, the stacking gel mixture was filled in the tubes and the water overlayed immediately. The tubes were left as such for about 50-60 min. The marker proteins used were BSA (monomer), ovalalbumin, cytocrome,carbonic anhydrase and phosphorylase.

The marker proteins as well as the sample solutions were dissolved in sample buffer and incubated at 37°C for 2 hours. A 25 ul sample was loaded to each gel tubes. The electrophoresis was carried out as a current of 5 mA/gel tube at 100 volts for 10 hours. The current was passed until the marker dye (bromophenol blue) was 1 cm from the anodic end of the gel. The gels were removed from the tubes by squirling water from a syringe between the gel and the glass wall.

(c) Gel Fixing, Staining and Destaining

After electrophoresis, the gel was kept in the fixer for 60 min. The staining was carried out by keeping the gel immersed for 30 minutes in the staining solution. The destaining

of gel was carried out in the solution containing isopropanol, glacial acetic acid and distilled water.

(e) Molecular Weight Determination

The migration distance of the tracing dye and of the blue protein zones from the top of the gel were recorded. Finally gels were stored in the destaining solution.

The relative mobility (Rf) of protein was determined by dividing the migration distance from the top of gel to the centre of protein band by the migration distance of bromophenol blue tracing dye from the top of the gel.

$$R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracing dye migration}}$$

The R_f values (as abscissa) were plotted against known molecular weights (co-ordinate on semi-logarithmic graph). The molecullar weights of unknown proteins were estimated with the help of a calibration curve.

Measurement of pH

All pH measurements were made on an Elico-EI-10 pH meter. Sodium tetraborate (0.1 M, pH 9.18) and potassium hydrogen pthalate (0.05 M, pH 4.0) solutions were used as standard buffer to calibrate the pH meter.

Spectrophotometric Measurements

Spectrophotometric measurements in the ultraviolet and visible range were carried out on a Bausch and Lomb spectronic-21 Spectrophotometer. All these measurements were carried out at room temperature.

3.3.5 IMMUNIZATION STUDIES

Immunization of Mice Against P. berghei Antigen Using 6,6' Trehalose Dimycolate (TDM) as an Adjuvant

The 6,6'-Trehalose Dimycolate (TDM), a mycobacterial glycolipid previously known as cord factor was obtained as a gift from Late Prof. E. Lederer, Laboratorie de Biochimie, C.N.R.S., Gifsuryvette, France.

Immunization Protocol

For immunization experiment, 100 healthy mice, (average weight 20-25 gm), were divided into four different groups. The immunization schedule and the amount of antigen protein/TDM administered to each group is shown in Table II.

In Group I each mouse received a total of 100 ug antigen protein + 500 ug TDM, on 0 day first injection (containing 50 ug antigen protein + 250 ug TDM) was given intraperitoneally. Subsequently, second injection, containing

TABLE II

Immunization Schedule

Immunization doses	No. of Animals	Antigen/TDM	
		0 Day (Ag/TDM)	10th Day (Ag/TDM)
Group I (100 ug Ag + 500 ug TDM)	25	50 ug/250 ug TDM	50 ug/250 ug TDM
Group II (500 ug TDM)	25	Nil/250 ug	Nil/250 ug TDM
Group III (100 ug Ag)	25	50 ug/Nil	50 ug/Nil
Group IV	25	Saline	Saline

All injections were given intraperitoneally

Ag: P. berghei antigen

TDM: 6,6'-Trehalose Dimycolate

Dose of inoculum = 0.2 ml

(50 ug antigen protein and 250 ug TDM) was given on the 10th day. Group II mice were immunized with 500 ug TDM. Mice in this group were injected with 250 TDM dose on zero day and 10th day. The amount of TDM given was distributed equally between the two injections. Group III mice received 100 ug antigen protein only. The injections were given to mice on zero day and 10th day, each inoculum having 50 ug antigen. Group IV contained only saline control mice. Each inoculum having antigen and TDM given alone, or in combination, contained 0.2 ml. This volume was maintained by the addition of saline. All immunizing doses were given via the intraperitoneal route.

3.3.6 TOTAL LEUCOCYTE COUNTS (TLC)

Total leucocyte counts were made according to the method described by Dacie and Lewis (1970). For the detection of circulating peripheral leucocytes, before and after immunization. The blood samples were collected in white blood cell counting pipettes upto 0.5 mark, and diluted with Turk's fluid (3% glacial acetic acid in distilled water) to which some methylene blue was also added. The leucocytes were counted in a leucocytometer.

3.3.7 SERUM ENZYME CHEMISTRY

The various serum enzyme studies in immunized

animals carried out were as follows:

A) Serum Glutamic Pyruvic Transaminase (SGPT)

B) Serum Glutamic Oxaloacetic Transaminase (SGOT)

C) Serum Acid Phosphatase

D) Serum Alkaline Phosphatase

3.3.8 DETECTION OF P. BERGHEI SPECIFIC ANTIBODIES

Antibody detection in immunized serum was carried out by means of several tests such as immunodiffusion (ID), indirect haemagglutination test and ELISA.

(A) Immunodiffusion

This test was performed as described previously. The central well was filled with antigen, while the peripheral wells received decomplemented antiserum. The slides incubated at room temperature were observed for the formation of precipited bands.

(B) Indirect Haemagglutination Test

Sheep erythrocytes (SRBC) were washed 4 times with phosphate buffered saline, pH 7.2. After final washing, 0.3% (V/V) suspension of RBC was prepared in isotonic phosphate buffered saline, pH 7.2. This suspension was mixed with an equal volume of 1/20,000 tannic acid and incubated

at 4°C for 30 min. with intermittant shaking. Tanned SRBC were washed thrice with PBS-BSA for the removal of the tannic acid and resuspended at 3% (V/V). Subsequently, an 0.1 ml tanned SRBC suspension was mixed with an equal volume of diluted antigen (100 ug/1 ml) and incubated at 37°C for 30 min. after intermittant shaking. The antigen coated SRBC were washed thrice with PBS-BSA and were later used for making 1.5% suspension. To each well of 'U' bottomed microtitre plate, .025 ml of a searially diluted serum sample from an immunized albino mice was added alongwith an equal volume of antigen coated RBC suspension. The control wells contained tanned RBC, normal sera and antigen. The plates were incubated at room temperature in a humid chamber for 1 hr. and kept overnight at 4°C. The highest dilution of the test serum giving a positive carpet like pattern was recorded as the titration end point.

(C) Enzyme - Linked Immunosorbant Assay (ELISA)

This test was performed according to the method of Lin et al. (1981) with slight modifications.

Preparation of Reagents

i) Coating buffer

An 0.05 M carbonate-bicarbonate buffer containing 0.02% sodium azide was prepared in distilled water. The

pH of the solution was adjusted at 9.6.

ii) Phosphate Buffer Saline - Tween 20

A solution containing 0.154 M PBS containing 0.05% Tween 20 (V/V) was prepared (pH 7.4).

iii) Diethanolamine Buffer

A 10% diethanolamine containing 0.01% $MgCl_2 . 6H_2O$ and 0.02% sodium azide was prepared (pH 9.8).

iv) Antimouse IgG (whole molecule) alkaline phosphate conjugate was used in the study.

v) Substrate

One tablet of p-nitrophenylphosphate was dissolved in 5 ml diethanolamine buffer and warmed at room temperature.

vi) Procedure

The ELISA test was performed in flat bottom microtitre plates. The plates were coated with 60 ug antigen protein in carbonate buffer and kept over night at 4°C. The plates were washed thrice with PBS/T buffer. Plates were then treated with 0.2 ml BSA solution (100 mg/100 ml) for 1 hr. and kept at 37°C. The plates were again washed and

treated with 0.2 ml of test serum in each well and incubated at 37°C for 2 hours and washed again. An 0.2 ml antimouse IgG alkaline phosphate conjugate (1:100 dilution with PBS/T) was added to each well. The plates were washed three times with PBS/T. An 0.2 ml substrate was added to each well. After 30 minutes incubation, the reaction was stopped by adding 0.05 ml of 3N NaOH. the colour intensity was measured after 15-20 minutes at 400 nm wave length read on ELISA reader (Series 700 Microplate Reader Cambridge Technology Inc. USA).

3.3.9 *ASSESSMENT OF CELL-MEDIATED IMMUNITY*

Leucocyte migration inhibition test and the delayed type skin hypersensitivity reaction (DTH) were employed for in vitro and in vivo assessment of CMI response.

(A) Leucocyte Migration Inhibition Test (LMIT)

LMIT was performed according to the method of DeLucca et al. (1982). The heparinized blood collected from immunized mice was incubated at 37°C for 1 hour with equal volume of 3.6% dextran solution in phosphate buffered saline (PBS, pH 7.2). Leucocyte rich plasma was collected and the leucocytes recovered by centrifugation at 200 g for 5 min. The pellet was washed thrice with Hank's Balanced Salt

Solution (HBSS). The leucocytes were resuspended in HBSS at a concentration of 4×10^7 cells/ml. The cell suspension was filled in hematocrit capillaries of equal size. The capillaries tubes were centrifuged at 100 g for 4 min. Using a sharp blade capillaries were cut at the cell fluid interface and fixed in pyrogen free perspex chambers. The chambers were filled with HBSS medium containing 5 percent foetal calf serum. The medium also contained penicillin, streptomycin and 1.5 ug P. berghei antigen per ml. The antigen was omitted in the control chambers. The chambers were covered carefully with coverslips so that no air bubble remained in the chambers. The chambers were incubated at 37°C for 24 hrs. in humid box. The area of migration in control as well as antigen chamber was recorded on a centimeter graph sheet with the help of a Camera Lucida and the area of migration calculated. The percent migration inhibition was calculated as follows:

$$\% \text{ Migration inhibition} = 100 - \frac{\text{Area of migration in antigen chamber}}{\text{Area of migration in control chamber}} \times 100$$

(B) Delayed Type Skin Hypersensitivity Reaction

Three weeks after complete immunization, all the control and test mice were skin tested for the appearance of delayed type skin hypersensitivity reactions. Two days

before the challenge, each mouse was inoculated intradermally with 10 ug of P. berghei antigen. Whereas on a seperate site 0.1 ml of normal saline was inoculated as a control sample. The test sites were checked at 48 hour periods. The diameter of indurated area was measured with the Vernier Calipper.

3.3.10 CHALLENGING THE ANIMALS

Thirty days after the last does, the immunized as well as control mice were challenged with 10^5 parasitized erythrocytes given intraperitoneally. After challenge the blood smears were prepared from tail tips and stained with Leishman. Slides were examined under microscope for counting percent parasitaemia.

For checking any subpatent infection, about 0.1 ml blood was taken from each recovered mouse and inoculated into fresh mice. Blood samples from the surviving mice belonging to group I and groups II were subinoculated into 25 fresh mice. Blood samples from the five survivors of group C were sub-inoculated into five mouse.

(A) Detection of Humoral Immune Response

The serum samples collected during post challenge period were tested for the production of specific antimouse

antibodies by enzyme linked immunosorbant assays as described earlier.

(B) Detection of Cell - Mediated Immune Response

The delayed type skin hypersensitivity reaction (DTH) were employed for the assessment of CMI responses.

3.4 HISTOPATHOLOGICAL STUDIES

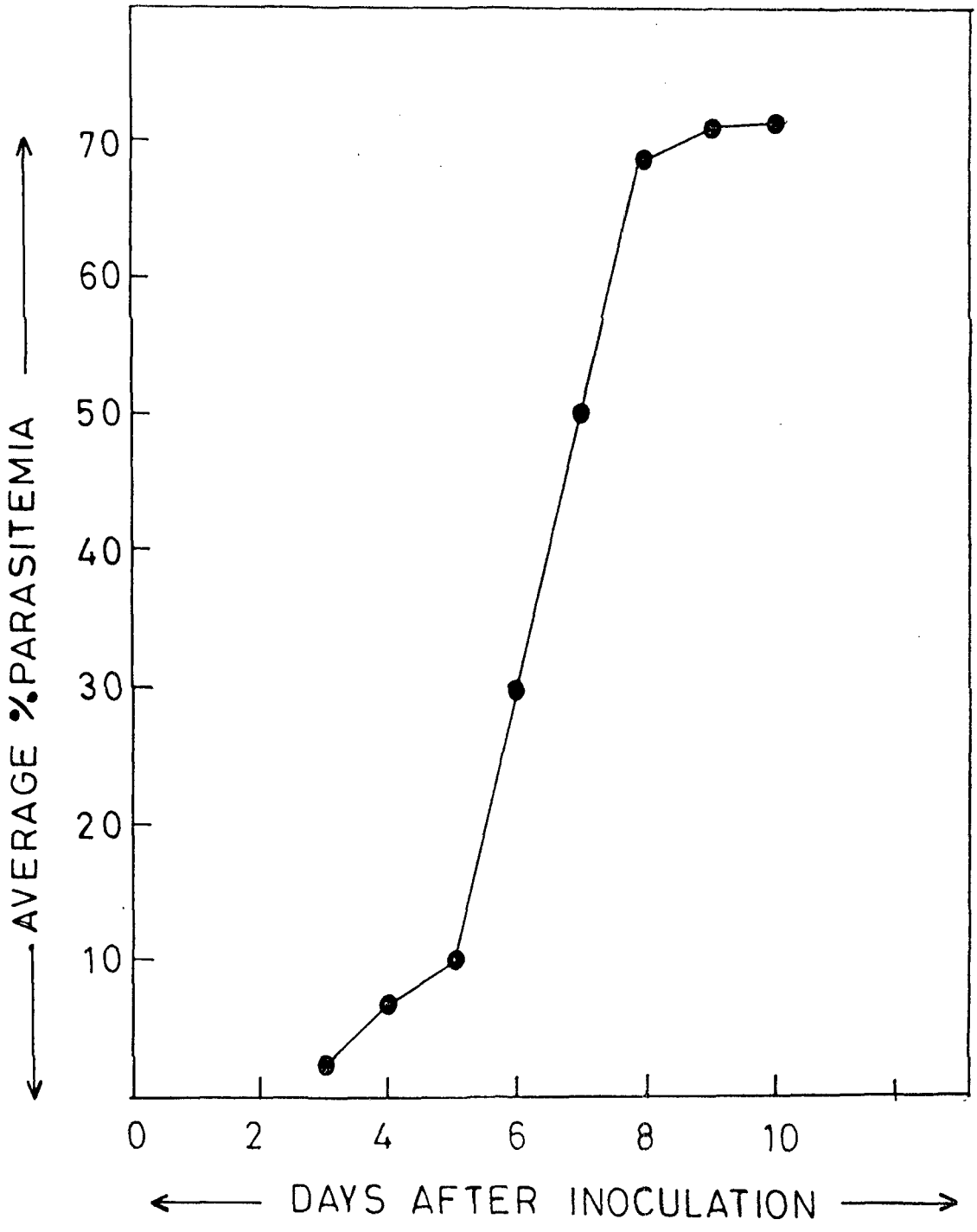
On sacrificing the animal, liver, spleen and kidney tissues were excised for sectioning. The organs were fixed in 10% formaline saline. The slides were stained with iron hematoxylin and eosin (H and E) and later examined microscopically.

4.1 MAINTENANCE OF PLASMODIUM BERGHEI

The P. berghei organisms were obtained from infected red blood cells using albino mouse as an animal model. The examination of blood smears from infected animals revealed that parasites started to appear in blood circulation usually on post inoculation day 3, following an intraperitoneal inoculation of 10^6 parasitized erythrocytes. The parasitaemia reached upto a maximum level of more than 80 percent on post inoculation day 8 (Figure 1, 2). The animals usually died after reaching a parasitaemic level of about 80 percent or more. The P. berghei (Strain, NK-65) was found to cause 100 percent mortality in white albino mice (Hissar Strain, India). Figure 3, shows the mortality curve of P. berghei infected mice. In a fully diseased state the animals were critically ill with staring hair. The hind legs and less often the fore legs, were paralyzed. The colour of the eyes changed to yellow, probably due to jaundice.

4.2.0 GROSS CHANGES IN P. BERGHEI INFECTED LIVER OF ALBINO MICE

At 60-70 parasitaemia, there was an increase in the volume of liver whose surface became granulated. In addition, the colour of liver changed to dark brown or even black at the peak parasitaemia.


AVERAGE %PARASITEMIA
70
60
50
40
30
20
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2
4
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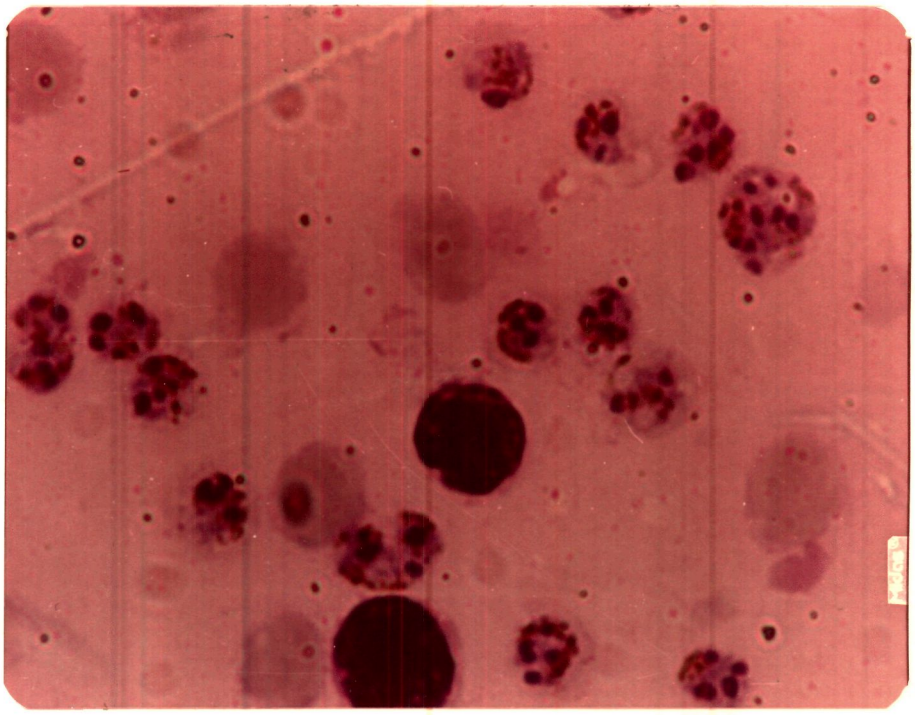
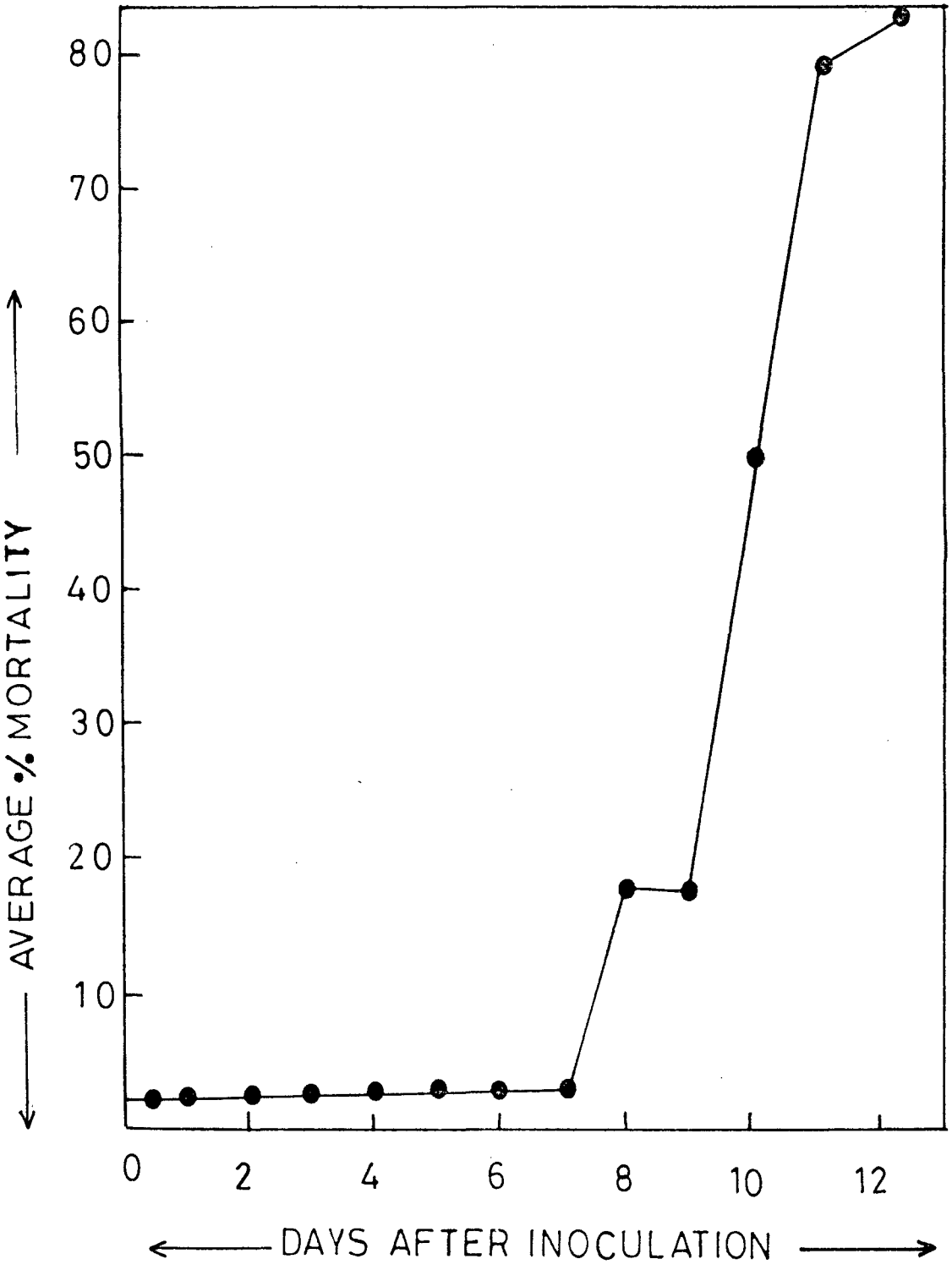
8
10
DAYS AFTER INOCULATION

Figure 2. Photograph showing Leishman stained *P. berghei* schizonts (x-1000).




80
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AVERAGE % MORTALITY
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2
4
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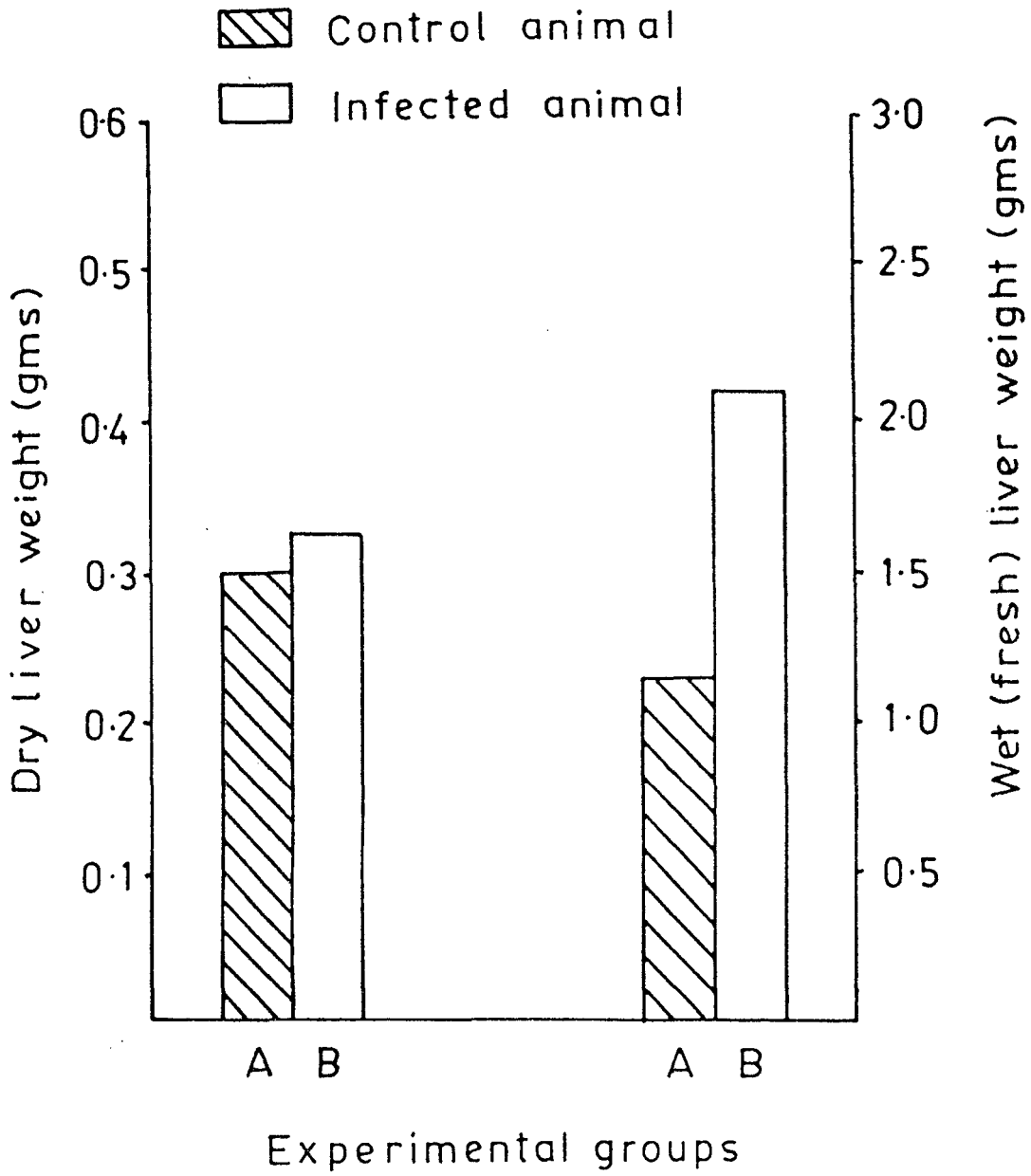
DAYS AFTER INOCULATION


Control animal
Infected animal
0·6
0·5
0·4
0·3
0·2
0·1
Dry liver weight (gms)
3·0
2·5
2·0
1·5
1·0
0·5
Wet (fresh) liver weight (gms)
A B
A B
Experimental groups

(i) Dry Weight of Liver

The change in dry weight of liver observed at high parasitaemia was unsignificant (Figure 4).

(ii) Wet Weight of Liver

Wet weight of liver of infected albino mice was found increased, significantly. The 'p' value was less than 0.001 ($P < .001$) (Figure 4).

4.3.0 BIOCHEMICAL STUDIES

Alterations in chemical constituents of Plasmodium berghei infected liver were analysed. Parallel analysis of normal liver was also carried out in order to obtain the control values. All the estimations were carried out in liver homogenates.

(i) Total Lipid

The results from our studies showed a significant increase in total lipid contents at 60-70% parasitaemia. The increase was observed in the order of (84.49%, $P < .001$) (Table III).

(ii) Total Cholesterol

Total cholesterol content of liver in P. berghei infected albino mice showed a slight but significant decrease.

Liver cholesterol contents decreased by (24%) at 60-70% parasitaemia (Table III).

(iii) Phospholipid

A similar fall in phospholipid levels in liver was also observed during the course of an infection in mice (Table III).

(iv) Lipid Peroxidation

A significant increase in production of lipid peroxidation was also observed due to oxidative damage of host liver. The increase in the lipid peroxidation was of the order of (314%) at 60 to 70% parasitaemia (Table III).

(v) Total Carbohydrate

The total carbohydrate content in the infected liver was found greatly decreased. Such depressed carbohydrate level was found statistically significant (78.76%, $P < .001$) (Table IV).

(vi) Total Glycogen and Glucose Content

Depletion of total carbohydrate content of liver during malarial infection was also associated with a rapid and

significant decrease of glycogen and glucose content of liver. The decrease in content of glycogen and glucose was observed to be of the order of (85.87%, $P < .001$; 78.6%, $P < .001$) respectively (Table IV).

(vii) Total DNA

Total DNA content was found decreased at high parasitaemia. The depletion was observed to be of the order of (79.58%, $P < .001$) (Table V).

(viii) Total RNA

Total RNA content was also found decreased at high parasitaemia. The decreased in the level of total RNA content was significant and in the order of (29.17%, $P < .001$) (Table V).

(ix) Total Protein

In the present study, a significant fall was observed in the protein level of infected liver. The decrease was statistically significant and percent change was found to be of the order of (57.71%, $P < .001$) (Table V).

(x) Liver Transaminase

Significant and rapid decrease in the liver glutamic pyruvate transaminase (GPT) and glutamic oxaloacetictransaminase

(GOT) was observed. Liver-GPT level decreased by (22%) while liver-GOT level decreased by (42.5%) at 60-70% parasitaemia (Table VI).

(xi) Liver Phosphatase

Liver phosphatase were increased significantly during severe malarial infection. Liver acid phosphatase increased by (75%), while alkaline phosphatase increased by (90%) at 60-70% parasitaemia (Table VI).

(B) Serum Analysis

(i) Serum Transaminase

Serum glutamic pyruvate transaminase (SGPT) level in P. berghei infected albino mice was elevated, showing an increase by (188%) at 60-70% parasitaemia. The increase was statistically significant ($P < .001$) Table VII.

Serum glutamic oxaloacetic transaminase (SGOT) level was also increased very rapidly and significantly by (84%) at high degree of parasitaemia (Table VII).

(ii) Serum Phosphatase

Serum acid and alkaline phosphatase levels in P. berghei infected liver of albino mice were increased significantly. The increase in acid phosphatase level was

TABLE III

Changes in Total Lipid, Cholesterol and Phospholipid and Lipid Peroxidation Content of Liver during *P. bergehi* Infection

Biochemical Asays	Control	Infected	Percent Change	'P' Value
Total Lipid	36.56 ± 3.58	67.45 ± 3.20	84.49	$P < .001$
Cholesterol	46.20 ± 3.62	35.25 ± 3.01	23.70	$P < .001$
Phospholipid	60.18 ± 3.01	48.20 ± 2.62	19.90	$P < .01$
Lipid Peroxidation	153.00 ± 28.40	650.00 ± 4.93	314.90	$P < .001$

Each value is the Arithmatic mean of six values ± S.D.

All values of $P < .01$ are significant. Significance was calculated by students's 't' test.

TABLE IV

Changes in Total Carbohydrate, Glycogen and Glucose Contents of Liver during *P. berghei* Infection

Biochemical Asays	Control	Infected	Percent Change	'P' Value
Total Carbohydrate (mg/g liver)	68.52 ± 5.10	14.55 ± 1.12	78.76	$P < .001$
Glycogen (mg/g liver)	45.54 ± 3.45	6.89 ± 2.10	84.87	$P < .001$
Glucose (mg/g liver)	30.85 ± 6.10	6.60 ± 0.98	78.6	$P < .001$

Each value is the Arithmatic mean of six values ± S.D.

All values of $P < .001$ are significant. Significance was calculated by student's 't' test.

TABLE V

Changes in Total DNA, RNA and Protein Contents of Liver during *P. berghei* Infection in Mice

Biochemical Assays	Control	Infected	Percent Change	'P' Value
Total DNA (mg/g liver)	4.84 ± 0.64	0.988 ± 0.10	79.58	$P < .001$
Total RNA (mg/g liver)	15.56 ± 4.0	11.02 ± 1.11	29.17	$P < .001$
Total Protein (mg/g liver)	176.003 ± 10.44	74.42 ± 2.395	57.71	$P < .001$

Each value is the Arithmatic mean of six values ± S.D.

All values of $P < .001$ are significant. Significance was calculated by student's 't' test.

TABLE VI

Changes in Glutamic Pyruvate Transaminase, Glutamic Oxaloacetic Transaminase, Acid Phosphatase and Alkaline Phosphatase Levels in Liver of Mice Infected with *P. berghei*

Biochemical Assays	Control	Infected	Percent Change	'P' Value
Liver Glutamic Pyruvate Transaminase (unit/mg tissue protein) (GPT)	155.30 ± 9.52	120.89 ± 10.6	22.00	$P < .001$
Liver Glutamic oxalo acetic transaminase (GOT) (unit/mg tissue protein)	44.8 ± 4.21	25.76 ± 2.27	42.5	$P < .001$
Liver acid phosphatase (unit/mg tissue protein)	0.116 ± 0.011	0.203 ± 0.018	75.0	$P < .001$
Liver Alkaline phosphatase (unit/mg tissue protein)	0.042 ± 0.008	0.080 ± 0.004	90.0	$P < .001$

Each value is the Arithmatic mean of six values ± S.D.

All values of $P < .001$ are significant. Significance was calculated by student's 't' test.

TABLE VII

Changes in SGPT, SGOT, Acid Phosphatase and Alkaline Phosphatase Levels in the Serum of *P. berghei* Infected Mice

Biochemical Assays	Control	Infected	Percent Change	'P' Value
SGPT (RF unit)	17.60 ± 2.50	49.41 ± 4.80	188	$P < .001$
SGOT (RF unit)	42.03 ± 4.82	77.45 ± 1.22	84	$P < .001$
Serum Acid Phosphatase (unit/mg protein)	0.64 ± 0.05	2.02 ± 1.10	215	$P < .001$
Serum Alkaline Phosphatase (unit/mg protein)	3.30 ± 1.82	6.58 ± 2.10	99	$P < .001$

Each value is the Arithmatic mean of six values ± S.D.

All values of $P < .001$ are significant. Significance was calculated by student's 't' test.

observed to be of the order of (215%), while alkaline phosphatase level, the increase was observed to be of the order of (99%) at 60-70% parasitaemia. P values were less than 0.001 (Table VII).

(C) Histochemical Studies

(i) Total Lipid

It was observed that in normal liver sections, the distribution of lipid was indistinct in the entire lobules (Figure 5). However, in liver sections from an infected specimen large number of blue - colour granules were observed. These lipid droplets were found in close association with the margins of the hepatocytes. Due to intense lipid infiltration, the hepatocytes showed degenerative changes (Figure 6).

(ii) Nucleic Acid

We have also carried out histochemical staining for identification of DNA and RNA contents at high parasitaemia. We have observed depletion in nucleic acid contents in infected individual. Small droplet like structures perhaps representing DNA and RNA were found in greater amount in control liver sections compared to infected ones (Figure 7,8).

Figure 5. Normal liver section showing absence of lipid droplets in lobules (H&E x 200).

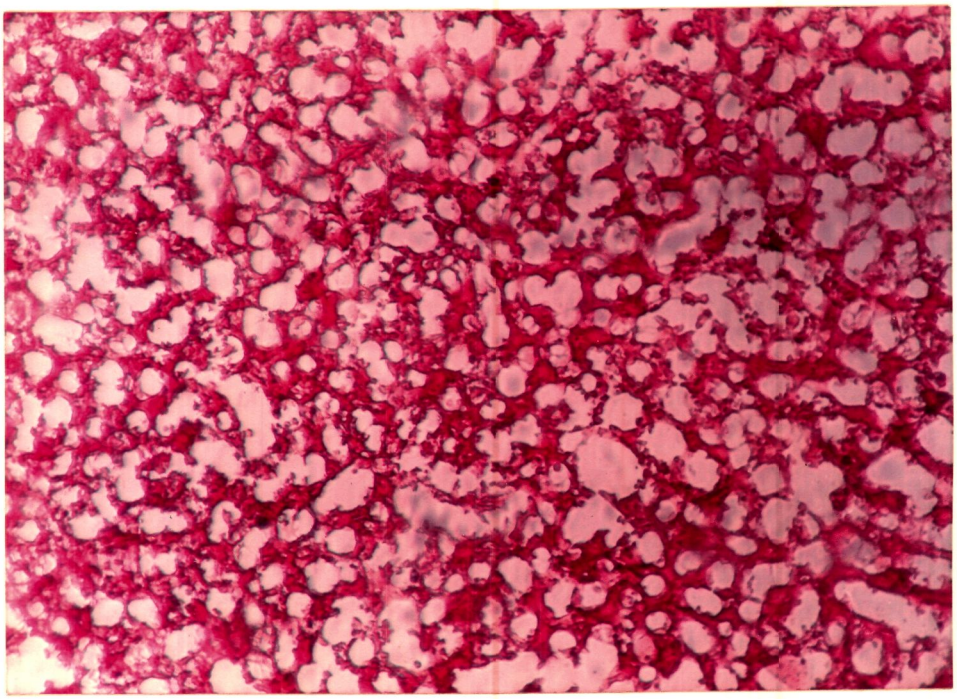


Figure 6. Liver section from infected animal showing distribution of lipid droplets in the lobules. hepatocytes showed degenerative changes (H&E x 200).

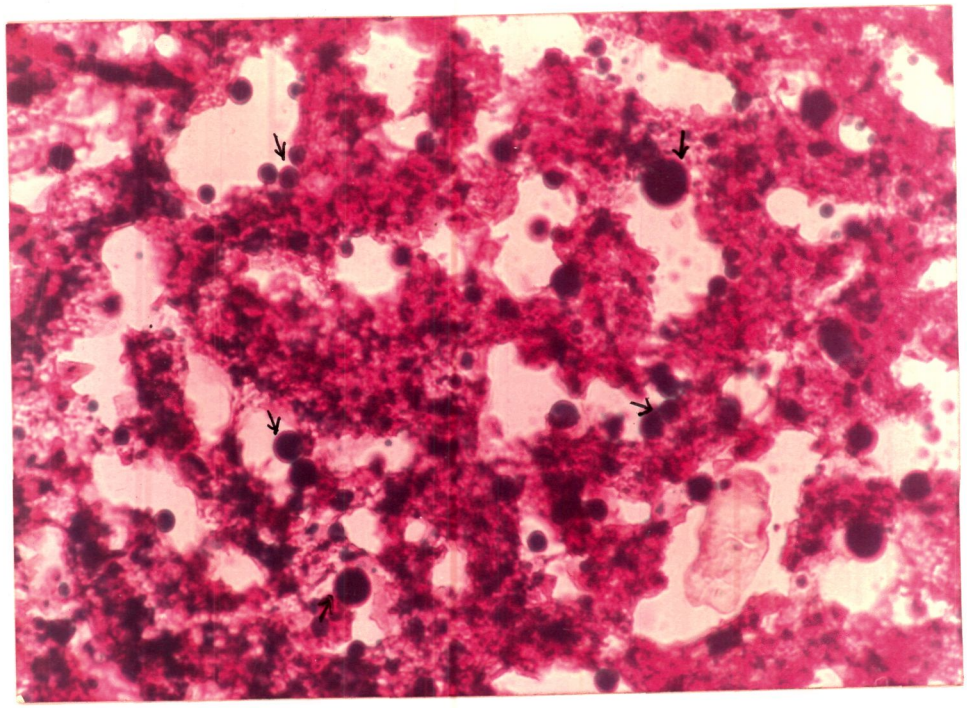


Figure 7. Normal liver section showing distribution of nucleic acid contents in hepatocytes (H&E x 100).

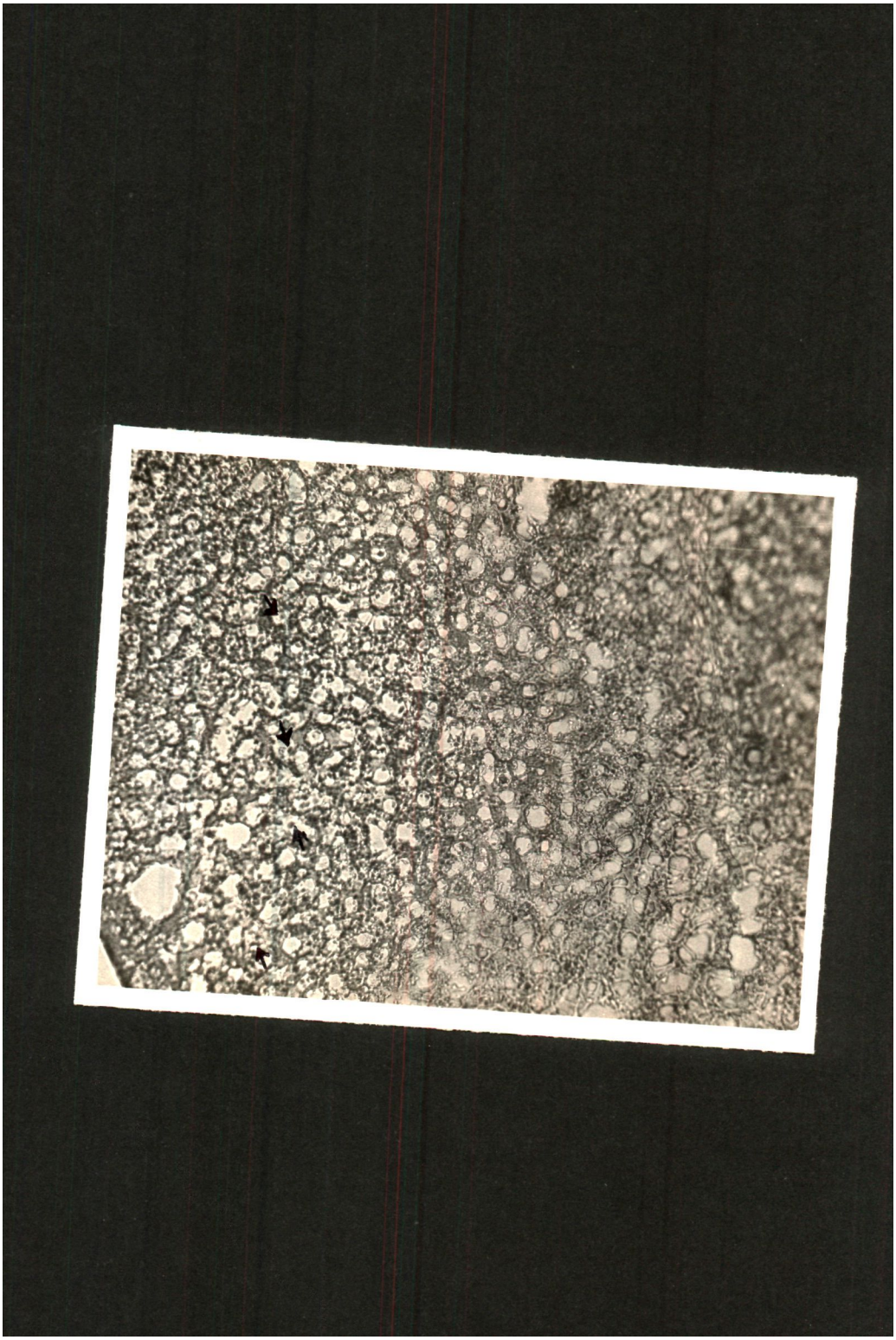
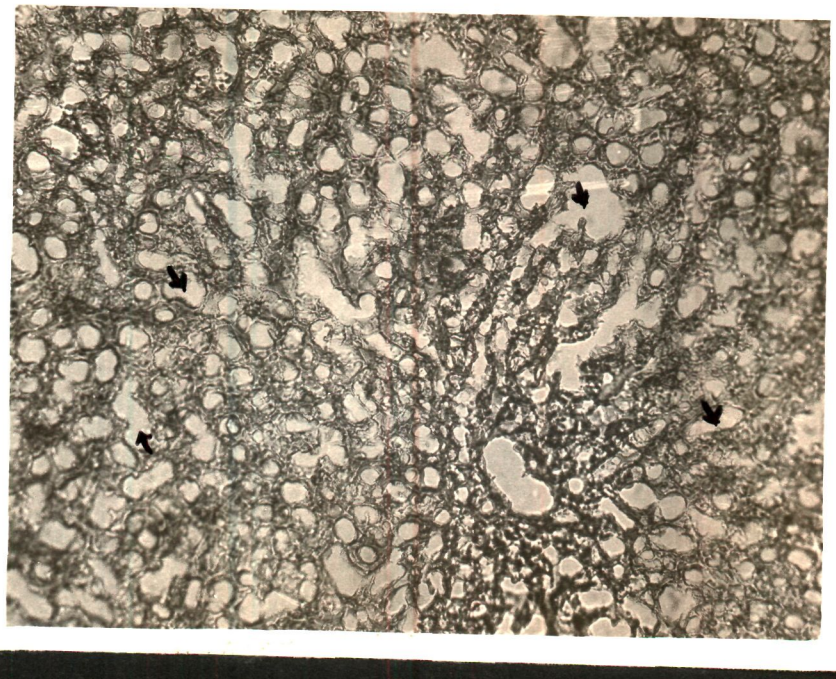


Figure 8. *P. berghei* infected liver section (60-70% parasitaemia) showing loss of nucleic acid content (H&E x 100).



4.4 IMMUNIZATION STUDIES

4.4.1 Isolation of Antigen

(A) Removal of Leucocytes

(a) About 85 percent leucocytes were removed by repeatedly washing the infected blood with chilled normal saline (Figure 9).

(b) Removal of leucocytes by passing whole blood through a column packed with α-cellulose and microcrystalline cellulose.

By this method about 98.5 percent leucocytes and 85 percent platelets were removed. Only about 10 ml samples of acid citrate dextrose treated blood can be passed efficiently through a 2 ml column containing equal parts of α-cellulose and microcrystalline cellulose. Percent wise removal of leucocytes was found proportionately decreased, if the larger quantities were passed through the column (Figure 10).

(B) Erythrocyte Lysis

Saponin was used to lyse the erythrocytes. Microscopic examination of released parasites after removal of erythrocyte debris showed that the harvested parasites were

considerably free from remnants of the host cell. Figure 11, shows the photomicrograph of free parasites.

(C) Purification of Parasites

The antigen suspension obtained after slow speed centrifugation was purified by passing it through density gradient centrifugation on Histopaque. The parasites formed a brown layer at the interface while the RBC contaminants formed the upper most layer.

No host erythrocyte contaminants were seen when Leishman stained smears of isolated parasite preparation were observed microscopically. Mostly parasites appeared intact. There was no apparent change in the morphology of parasites. Figure 9, shows a Leishman stained smear of purified parasite preparation.

(D) Preparation of Soluble Antigen

The RBC free parasite were further disrupted by ultrasonication of parasite suspension in chilled normal saline (Figure 12). The supernatant thus obtained after centrifugation was collected and used as soluble antigen.

4.4.2 CHECKING THE PURITY OF ANTIGEN

The purity of P. berghei antigen obtained on

Histopaque did not appear to contain any mouse RBC contaminants. This was confirmed biochemically (PAGE) and immunologically against normal mouse RBC antiserum in immunodiffusion and counter-immunoelectrophoresis.

Detection of Antibody titre in Normal mouse RBC Antiserum

Normal mouse RBC antibodies were detected by indirect haemagglutination test (IHA). An antibody titre of 1:1280 was detected in sera samples obtained from immunized animals at the end of 4th week. Only few normal mouse RBC antibodies were detectable in the first week. But at the end of 2nd and 3rd week, antibody titres were detected as 1:320 and 1:640 respectively (Table VIII).

(A) Immunodiffusion

(i) Figure - 13, shows an immunodiffusion plate with normal mouse erythrocyte and P. berghei antigen at peripheral wells with antinormal mouse erythrocyte serum at the centre. A precipitin band was observed between the normal mouse erythrocyte and antinormal mouse erythrocyte serum.

(ii) Figure - 14, shows an immunodiffusion plate with normal mouse erythrocyte and P. berghei antigen along with anti- P. berghei serum at the centre. The precipitin bands were observed between P. berghei antigen and anti P. berghei serum.

(B) Counter Immuno-Electrophoresis

Results obtained after counter immuno-electrophoresis were found more or less similar to immunodiffusion.

(i) No precepitin bands were formed between P. berghei antigen and anti-normal mouse serum (Figure 15).

(ii) Similarly normal mouse erythrocytes extracts did not react with anti P. berghei serum (Figure 16).

(C) Purification of Isolated Antigen (Poly Acryl Amide Gel Electrophoresis)

Isolated antigen and normal mouse erythrocyte preparation were run on PAGE. Comparision of bands obtained on PAGE showed that the antigen preparation isolated by density gradient centrifugation was completely free from host erythrocyte contaminants (Figure 17).

4.4.3 BIOMOLECULE ESTIMATIONS

Protein concentration of soluble antigen was estimated as 2,500 ug/ml protein. The concentration of RNA and DNA per ml of antigen preparation was 400 ug/ml and 200 ug/ml respectively, the hexoses concentration of soluble antigen was 200 ug/ml (Table IX).

TABLE VIII

Antibody Titres in Weekly Sera Samples from Rabbits Immunized AgainstNormal Mouse RBC Antigen

Weeks	Reciprocal of Precipitin Titres
Ist Week	20
IInd Week	320
IIIrd Week	640
IVth Week	1,280

Figure 9. Photograph showing smear from an infected blood made after leucocyte removal by slow speed centrifugation (x-1000).

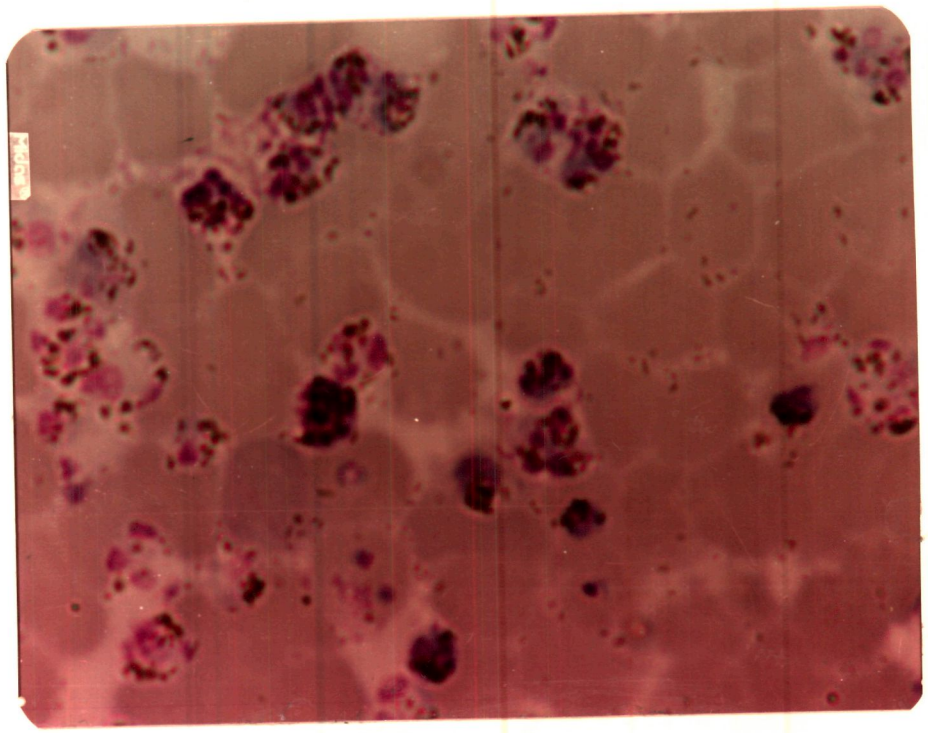


Figure 10. Removal of leucocytes from blood by α-cellulose and microcystalline packed column.

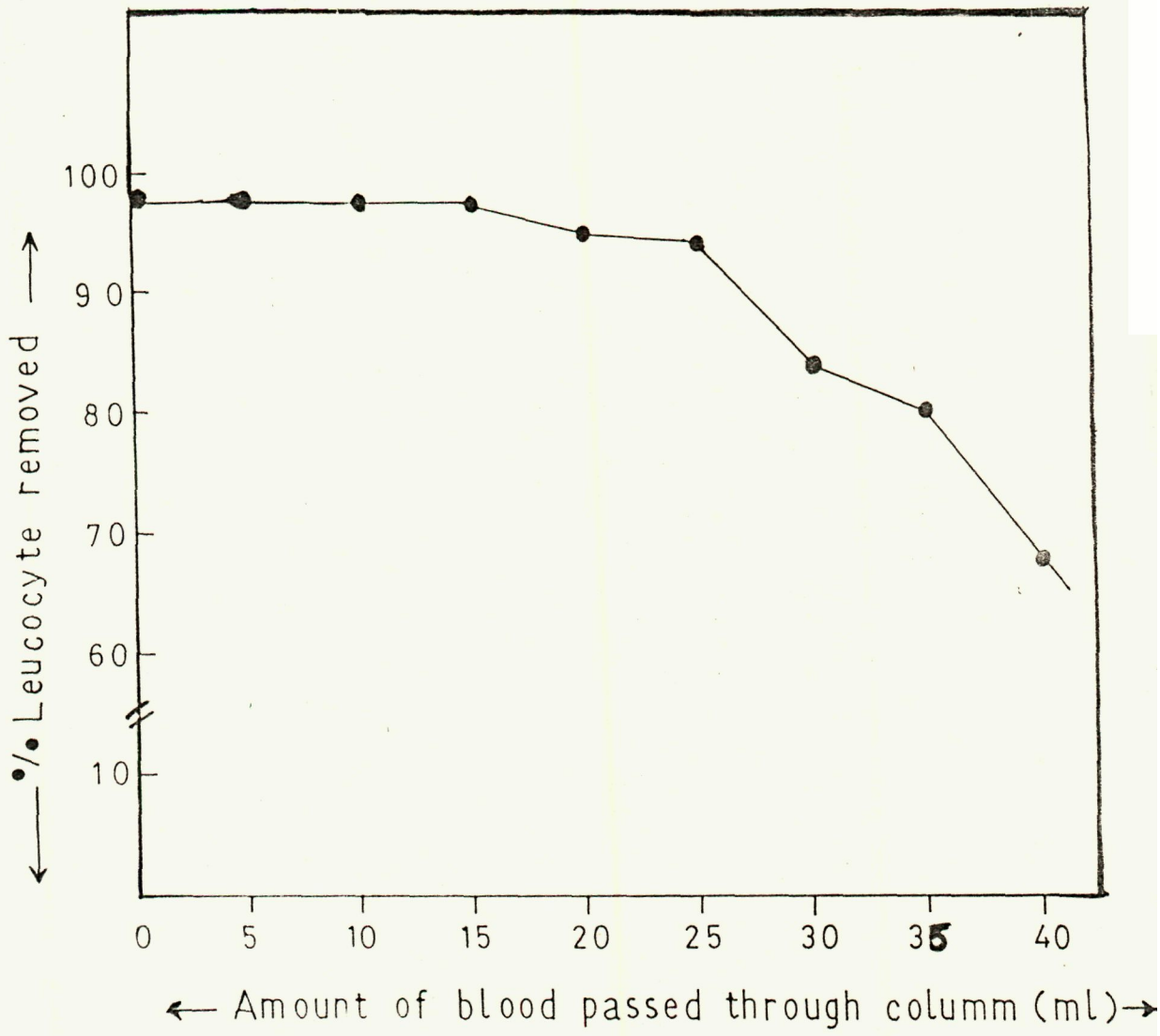

100
90
80
70
60
10
% Leucocyte removed
0
5
10
15
20
25
30
35
40
Amount of blood passed through columm (ml)

Figure 11. Photograph of saponin freed P.berghei organisims (x-1000).

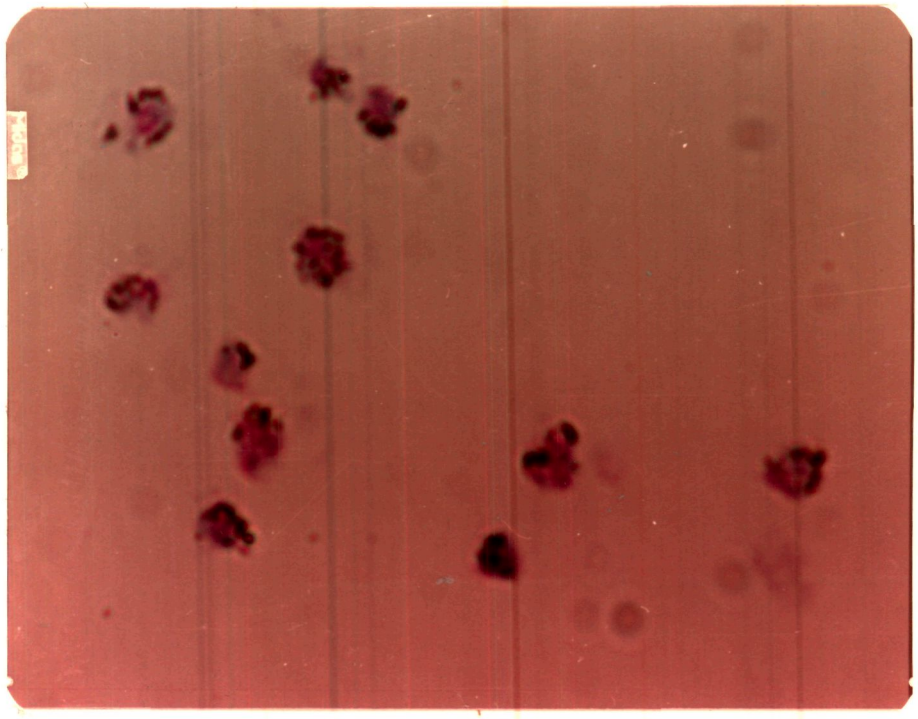


Figure 12. Photograph of P. berghei disrupted parasites after sonication (x-1000).

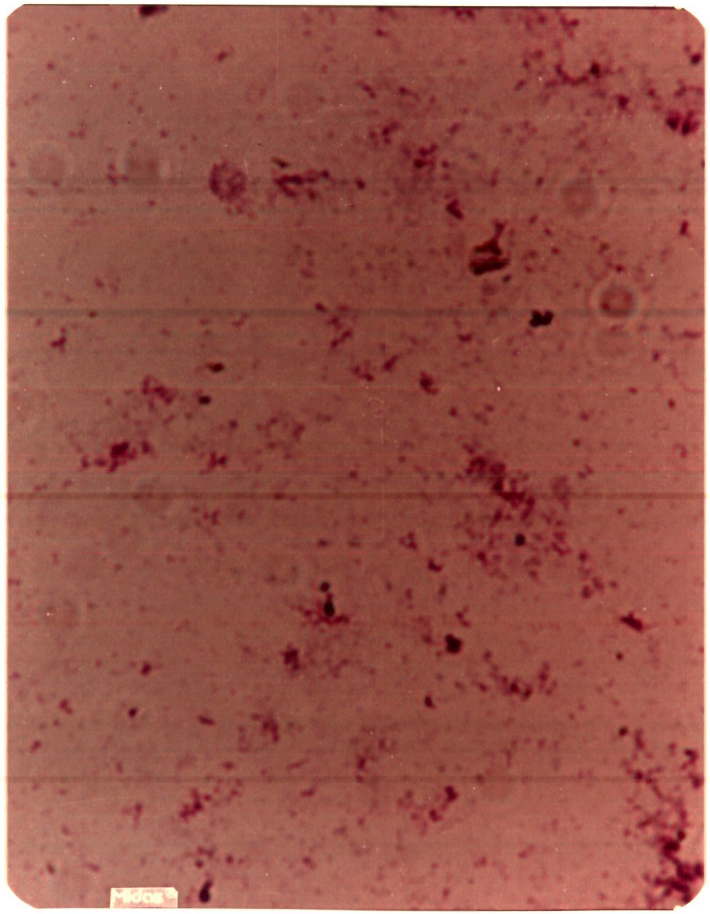


Figure 13. Immunodiffusion plate showing precipitin bands between antierythrocyte serum and normal mouse erythrocyte exract.

The reaction was performed in 0.8% agarose gel in 0.05 M veronal buffer pH 8.6.

ANME : Anti mouse erythrocyte serum raised in rabbits.

Pb : P.berghei antigen.

NME : Normal mouse erythrocyte extract.

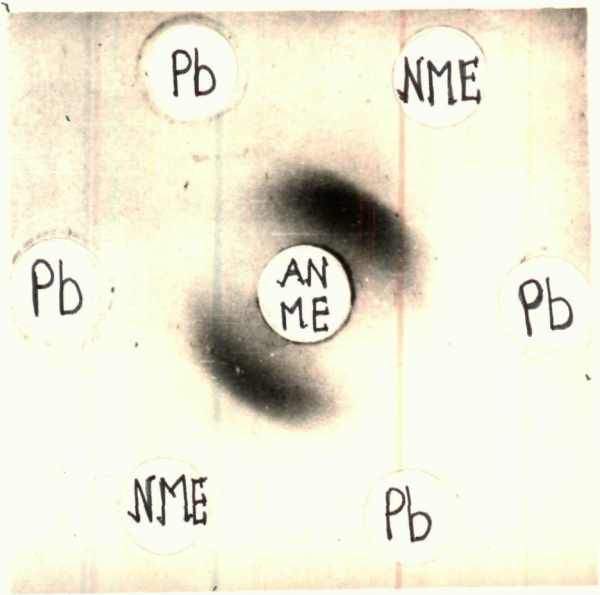

Pb
NME
Pb
AN
ME
Pb
NME
Pb

Figure 14. Immunodiffusion plate showing precipitin bands between P. berghei antigen and anti P. berghei serum.

The reaction was performed in 0.8% agarose gel in 0.05 M veronal buffer pH 8.6.

APb : Anti P. berghei antigen.

NME : Normal mouse erythrocyte extract.

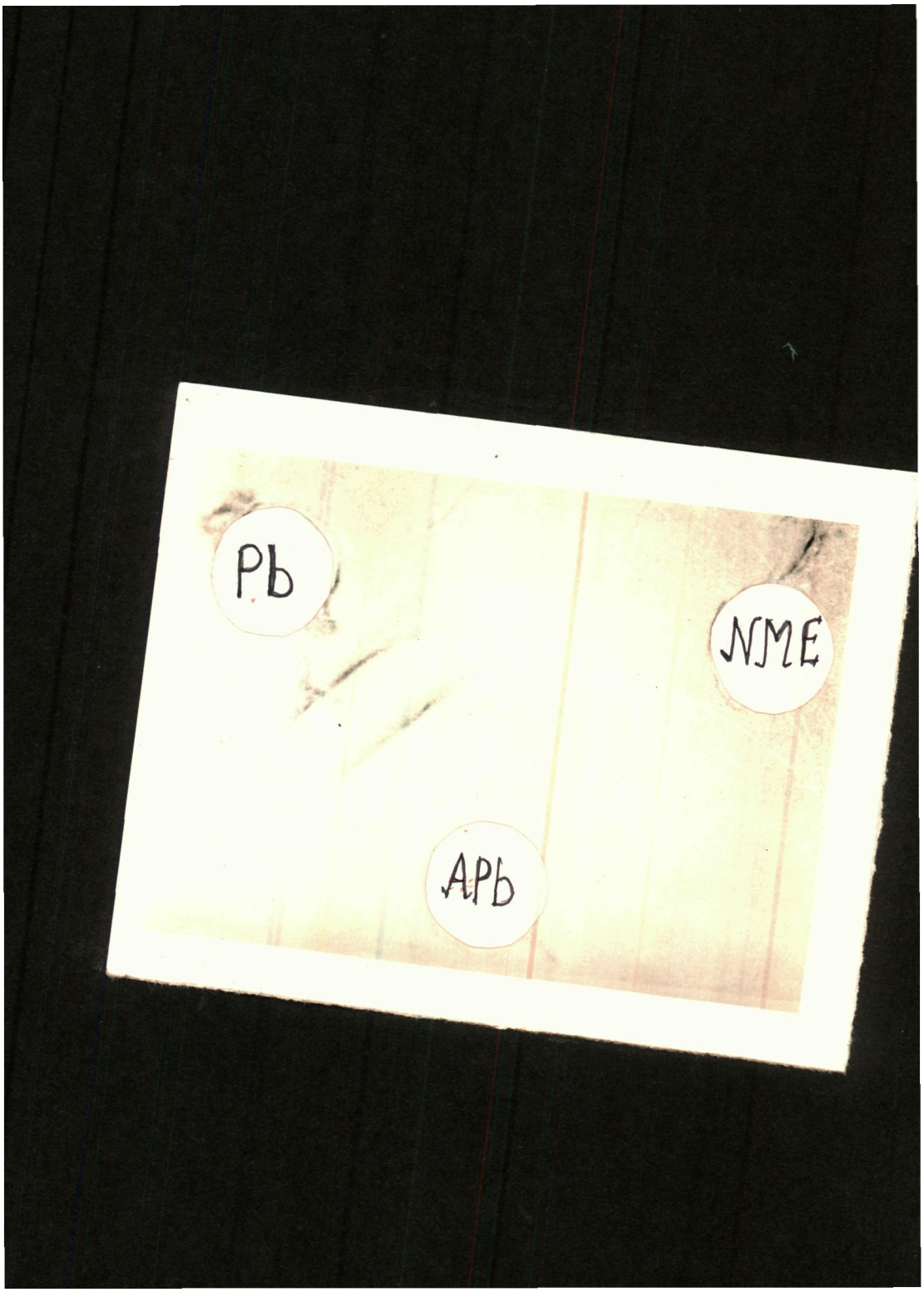

Pb
NME
APb

Figure 15. Counter immuno electrophoresis plate showing reaction between P. berghei antigen and antinormal mouse erythrocyte serum.

The reaction was performed in 0.8% agarose gel in 0.05 M veronal buffer pH 8.6. The current was 7 m Amp/slide.

Pb : P. berghei antigen.

ANME : Anti normal mouse erythrocyte serum raised in rabbits.

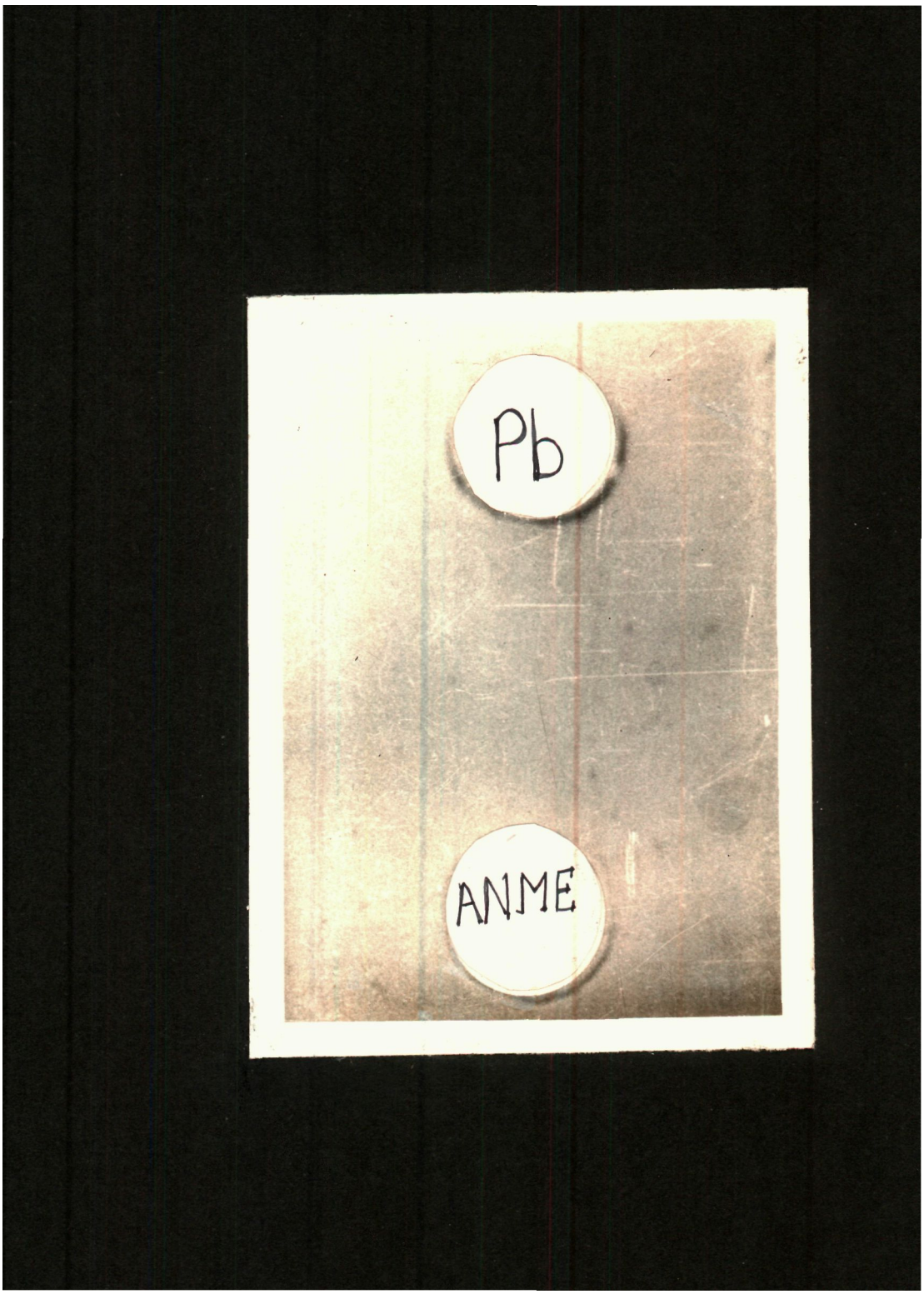

Pb
ANME

Figure 16. Counter immuno electrophoresis plate showing reaction between normal mouse erythrocyte extract and anti P. berghei serum.

The reaction was performed in 0.8% agarose gel in 0.05 M veronal buffer, pH 8.6. The current was 7 m Amp/slide.

NME : Normal mouse erythrocyte extract.

APb : Anti P. berghei serum.

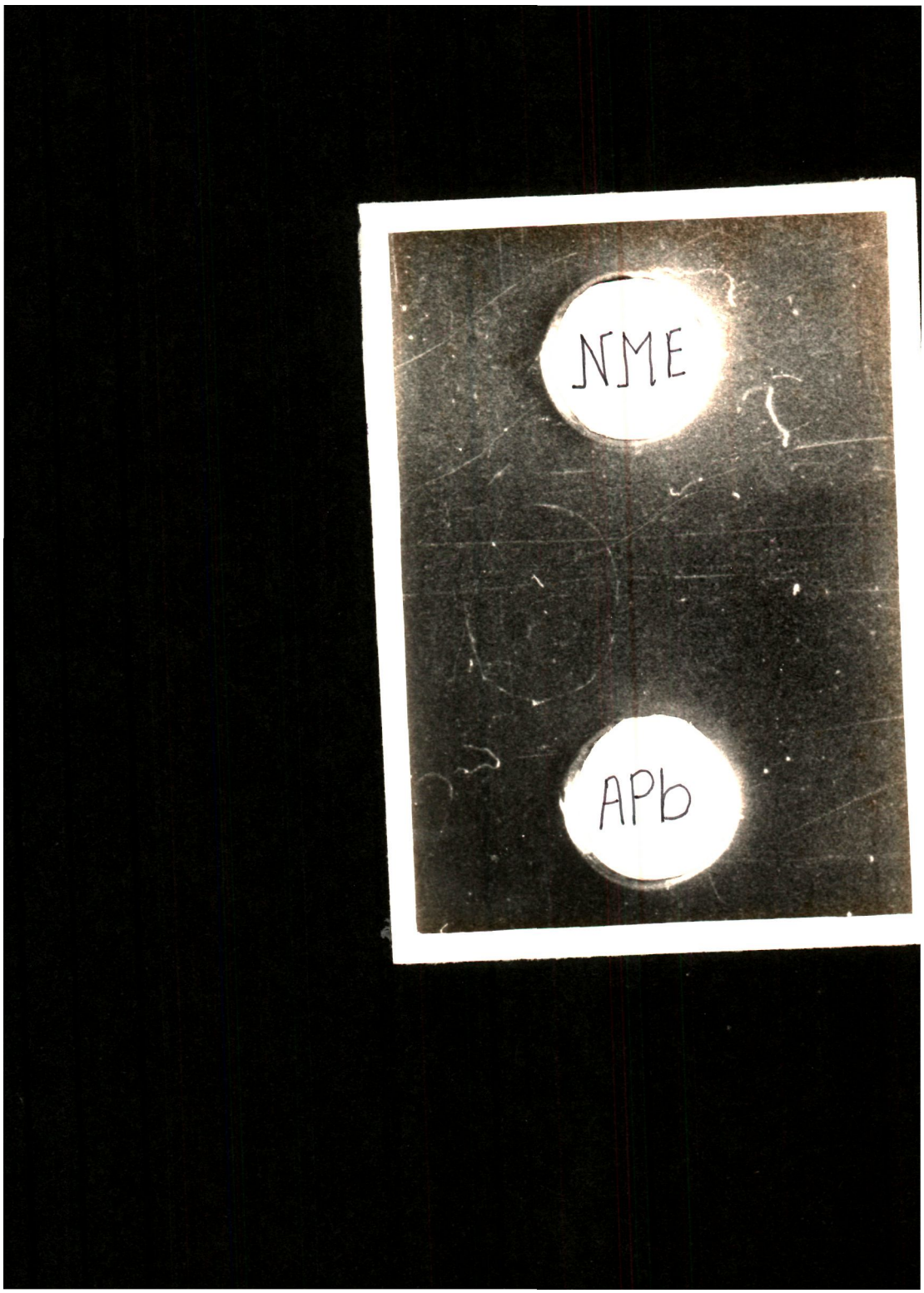

NME
APb

Figure 17. Polyacrylamide gel rods showing:

a) Normal mouse RBC proteins.

b) *P. berghei* proteins obtained after electrophoresis.

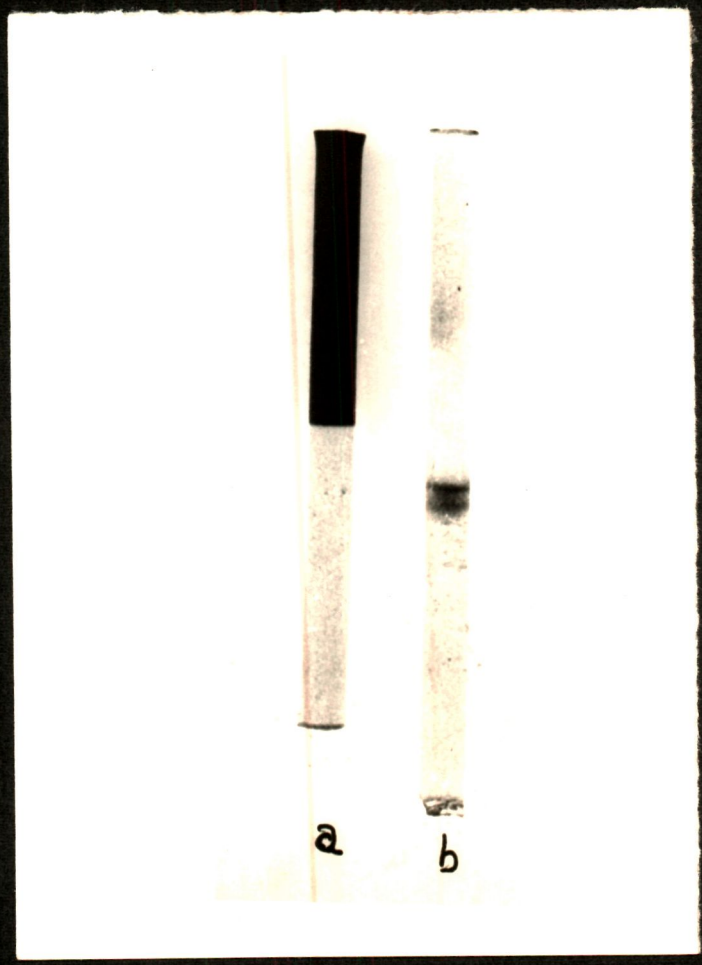

a
b

TABLE IX

Biomolecule Estimations in *P. berghei* Antigen Preparation

Antigen Preparation (Biomolecule Estimations)	Amount/ml. (ug)
Protein Concentration	2,500
RNA Concentration	400
DNA Concentration	200
Hexoses Concentration	200

4.4.4 CHARACTERIZATION OF P. BERGHEI ANTIGEN

The SDS-runs of the soluble extracts, showed eleven protein bands in the molecular weight range of M_r 12,000-150,000 daltons. Figure 18, showed the SDS-PAGE profiles of both the antigen preparations and that of lysed mice RBC.

4.4.5 TOTAL LEUCOCYTE COUNT

There was an overall increase in the total leucocytes count of the immunized animals. The increase in total leucocyte count was highest in animals immunized with antigen in combination with TDM. Thhe leucocyte counts/mm^3 were also increased in animals injected with antigen or TDM alone, though it was comparatively lower (Table X).

4.4.6 BIOCHEMICAL ASSAYS IN IMMUNIZED ANIMALS

Due to a continuous destruction of the liver cells several biochemical estimations were carried out in the serum of immunized animals. During immunization it was found that these enzymatic levels first increased to some extent and then came nearer to the control values, after the completion of immunization. Figure 19, show that levels of both the serum transaminase e.g. SGPT and SGOT returned to normal values after 15 days of completion of immunization.

Similarly we have also estimated acid and alkaline phosphatase levels in serum, which showed that the levels of acid and alkaline phosphatase were found nearer to control values about 15-16 days after completing the immunization (Figure 20).

4.4.7 DETECTION OF P. BERGHEI SPECIFIC ANTIBODIES

(a) The results of immunodiffusion showed the presence of precipitin lines between the antigen and sera samples from group I and III. The sera samples from other groups showed no precipitin lines (Figure 21). These bands showed the development of P. berghei specific antibodies in the sera of mice belonging to group I and III.

(b) Indirect Haemagglutination Tests (IHA)

With the serum samples of group I (Ag + TDM), the range of reciprocal IHA titres reported was 256 to 1024. The range of reciprocal IHA titre was 32-128 with serum samples of group III (Ag alone). For the remaining two groups, the highest titre value was 32 which was arbitrarily taken as negative. This showed that sera of mice belonging to group-I and group-III were positive for P. berghei antigen (Table XI).

(c) ELISA Test

Animal groups immunized with antigen plus TDM

combination showed greatly enhanced level of anti - P. berghei antibodies. Some what higher levels of P. berghei specific antibodies were also recorded in group-III mice which were immunized with antigen alone. Antibodies specific to P. berghei were not detected in the sera of mice belonging to other groups (Table XI).

4.4.8 DETECTION OF CELL MEDIATED IMMUNE RESPONSE

To determine the cellular immune response, we conducted assays using leucocyte migration inhibition test, and delayed type skin hypersensitivity reactions.

(A) Leucocyte Migration Inhibition Test (LMIT)

The results of leucocyte migration inhibition tests are shown in Table XII. Only the migration of leucocytes from group -I animals was inhibited in the presence of specific antigen (percent migration 68.7). No such inhibition was observed in test animals belonging to group-II, III and IV. This showed the development of cell mediated immunity in group I animals.

(B) Delayed Type Skin Hypersensitivity Test

The results of skin hypersensitivity reactions are shown in Table XIII. Skin reactions in animals sensitized with

antigen TDM combination (Group-I) varied from simple wheal and flare reaction to a well developed zone of erythema with induration.

The animals in group-II, III, IV showed very weak reaction (Table XIII) indicating the absence of a proper CMI response. This showed the development of cellular immunity in group-I mice (Figures 22, 23).

4.4.9 POST CHALLENGE STUDIES

In our immunization studies, animals, which were immunized with antigen plus TDM combination and animals which were injected with TDM alone showed 100 percent protection. Group III animals, immunized with Ag alone showed 20 percent protection on the other hand, the mice in group IV showed 100 percent mortality (Table XIV). To determine the degree of protection in albino mice, we had taken into consideration the prepatent period, the magnitude of peak parasitaemia and survival rate during the post challenge period.

Maximum prepatent period of 9 days was observed in mice receiving 100 ug antigen with 500 ug TDM in group I. Animals belonging to group II, injected with 500 ug TDM, showed 6 days prepatent period. Group III animals, inoculated with 100 ug antigen showed prepatent period of 4 days and mice from control group showed a prepatent period of 3 days (Table XIV).

Group I animals immunized with Ag + TDM showed peak parasitaemia (1.5±0.25). An average peak parasitaemia of 4 percent was recorded in group II animals receiving 500 ug TDM only. Parasitaemia reached upto an average of 20 percent in mice immunized alone with antigen. In saline control group IV, almost all the mice expired reaching an average parasitaemia of 78 percent of higher (Figure 24).

All those mice which were survived with first challenge were rechallenged after two months. Twenty five age matched uninfected animals were also infected at the same time, which served as control animals. Blood smears were prepared daily from all the survived animals and control animals to observed the appearence of malaria parasites. All the animals which were treated as control died within eight to ten days, while no parasites were detectable in the blood smears of animals which were rechallenged (Table XV).

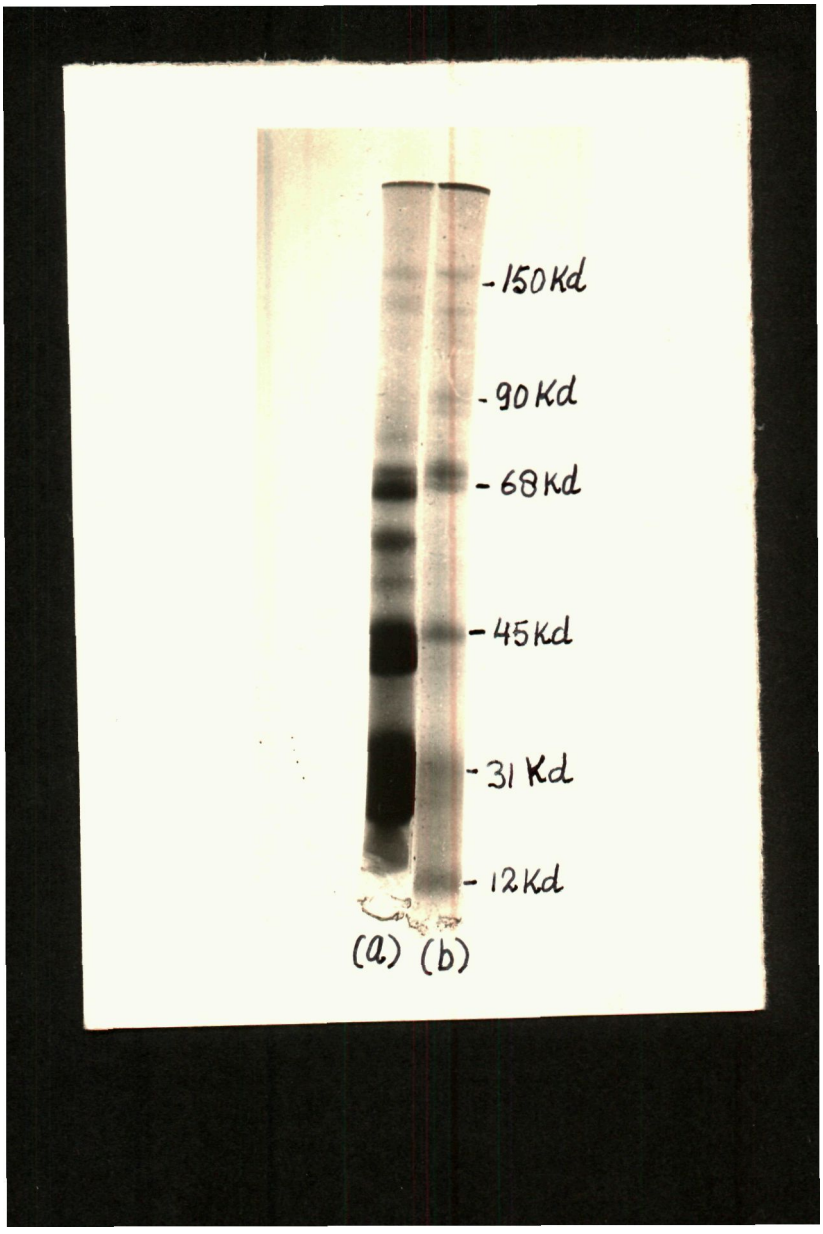
Humoral and Cell Mediated Immune Response

Humoral and cell mediated immune responses against P. berghei antigen, during post challenge studies were also measured. Anti -malarial antibodies were detected in all the groups. Although their titre values were very low except Ag +TDM group and Ag group only. The titre values were detected by ELISA, the highest titre was found as 2048 in Ag + TDM group (Figure 25).

Figure 18. Sodium dodecyl polyacrylamide gel electrophoresis showing:

b) Protein bands isolated from soluble extract of P. berghei.

a) Normal mouse erythrocyte protein.


150Kd
90Kd
68Kd
45Kd
31Kd
12Kd
(a)
(b)

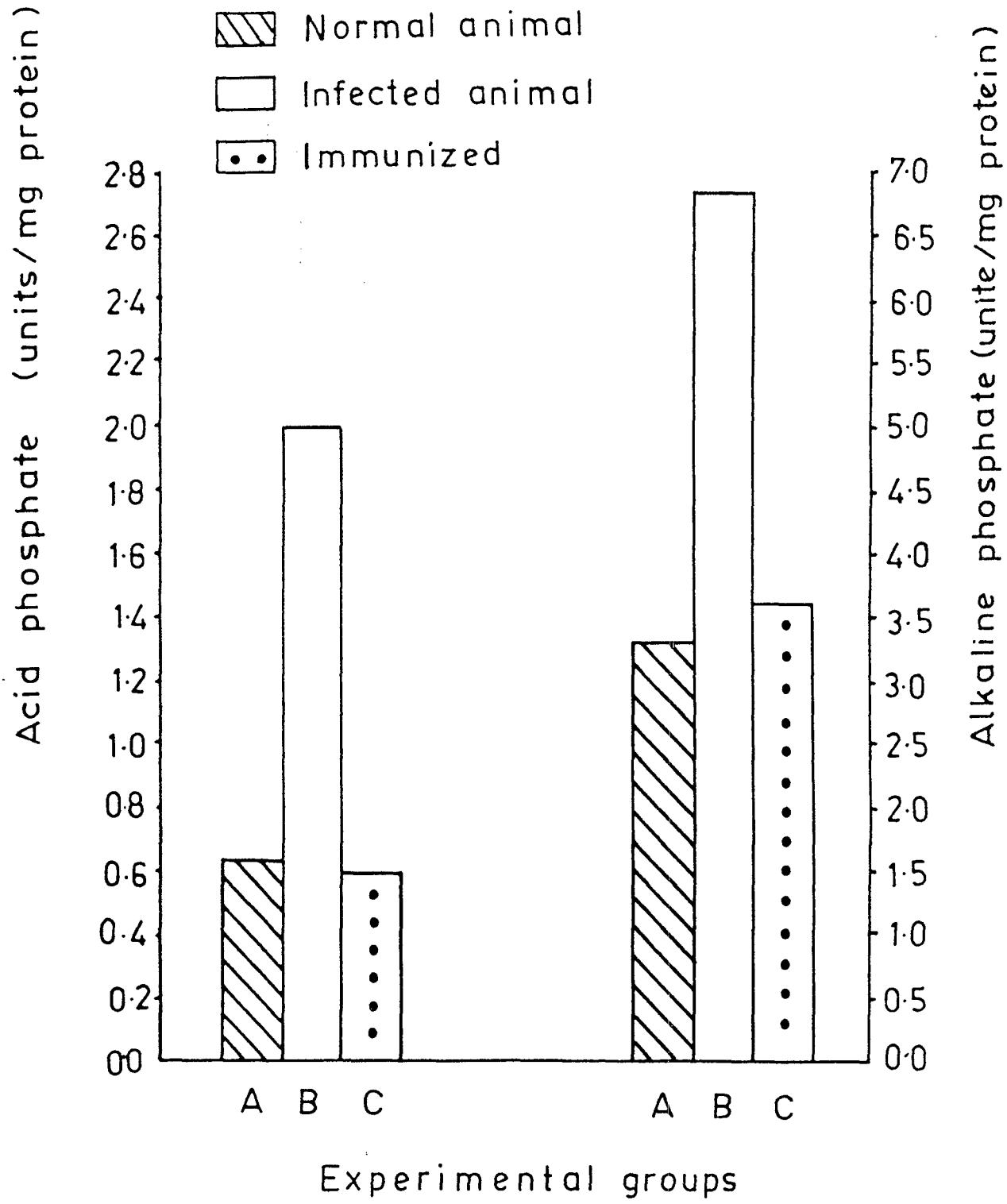

Normal animal
Infected animal
Immunized
Acid phosphate (units/mg protein)
Alkaline phosphate (unite/mg protein)
2·8
2·6
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
7·0
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
2·5
2·0
1·5
1·0
0·5
0·0
A B C
A B C
Experimental groups

TABLE X

Total Leucocyte Count

Immunization group	Total Leucocyte (Mean ± S.D.) Count/mm^3	
	Before Immunization	After Complete Immunization
Group - I (100 ug Ag + 500 ug TDM)	19430 ± 1200	31300 ± 850
Group - II (500 ug TDM)	13840 ± 2010	20520 ± 1800
Group - III (100 ug Ag alone)	20380 ± 2100	25800 ± 2500
Group - IV	24230 ± 1190	24000 ± 1700

Ag: P. berghei antigen, TDM = 6,6' Trehalose Dimycolate.

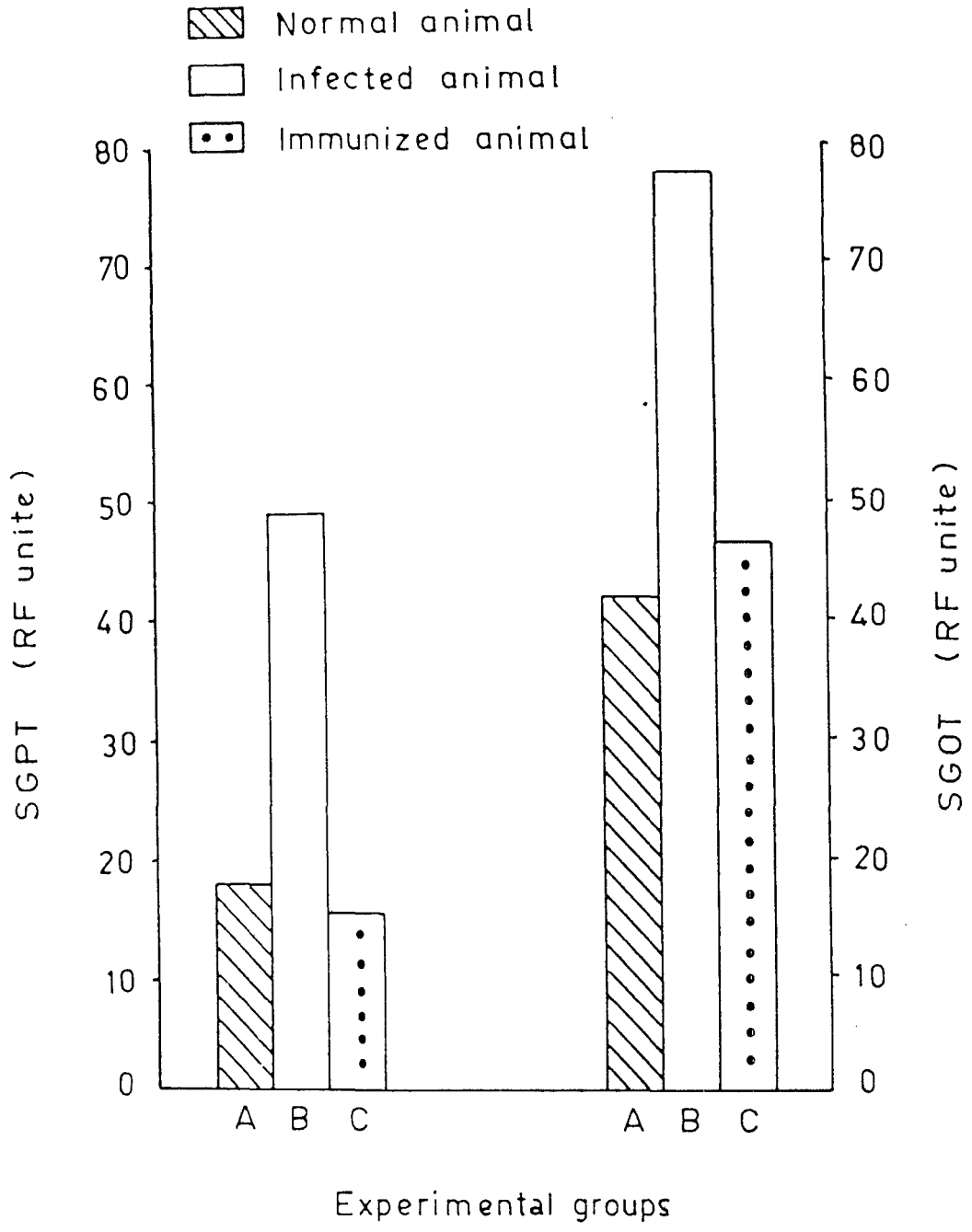

Normal animal
Infected animal
Immunized animal
SGPT (RF unite)
SGOT (RF unite)
0
10
20
30
40
50
60
70
80
A B C
A B C
Experimental groups

Figure 20. Figure showing acid and alkaline phosphatases levels from, normal, infected and immunized animals.

Figure 21. Immunodiffusion plate showing precipitin band between P. berghei antigen in Central and antisera obtained from immunized animals of Group I and Group III. Group II and Group IV animals had no precipitin bands with antigen.

Group I : Ag plus TDM

Group II : TDM alone

Group III : Ag alone

Group IV : Saline control.

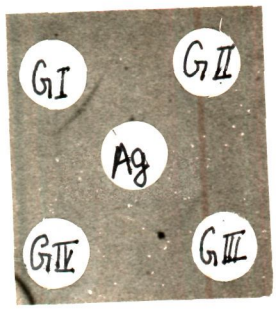

GI
GII
Ag
GIV
GIII

TABLE XI

Result from IHA and ELISA Tests

Immunization Group	IHA Tests (Range of reciprocal titre)	ELISA
Group - I (100 ug Ag + 500 ug TDM)	512 ± 363 (256 - 1024)	1 : 1024
Group - II (500 ug TDM)	32 ± 22.62 (16 - 64)	1 : 32
Group - III (100 ug Ag)	64 ± 45.25 (32 - 128)	1 : 128
Group - IV (Saline)	Negative	Negative

Ag - P. berghei antigen

TDM - Trehalose Dimycolate

1: 100 dilution of sera used in ELISA

The IHA titres are the Arithmatic mean of 25 values ± S.D.

Titres value less than 32 were taken as negative.

The value given in paranthesis indicates the range of IHA titres.

TABLE XII

Leucocyte Migration Inhibition Test

Immunization group	Percent migration	Percent migration inhibition
Group - I (100 ug Ag + 500 ug TDM)	68.7	31.3 ± 3.2
Group - II (500 ug TDM)	97.7	2.30 ± 0.56
Group - III (100 ug Ag)	92.5	7.5 ± 1.75
Group - IV (Saline)	100.00	0.00

Ag = P. berghei antigen

TDM= Trehalose Dimycolate

TABLE XIII

Results of Delayed Type Hypersensitivity Test Reaction

Immunization Group	Amount of Antigen injected (ug)	Reaction to skin test after 48 hrs.	
		Skin reaction diameter (mm ± S.D.)	Skin induration (mm ± S.D.)
Group - I	10	11.2 ± 0.73	1.58 ± 0.21
Group - II	10	2.2 ± 0.33	0.69 ± 0.39
Group - III	10	4.0 ± 1.07	0.71 ± 0.69
Group - IV	10	1.0 ± 0.12	0.45 ± 0.35

Ag = P. berghei antigen

TDM = Trehalose Dimycolate

Mean = S.D.

Figure 22. The site of intradermal skin inoculation in the absence of delayed type reaction.

Figure 23. Photograph showing the appearence of a delayed type skin hypersensitivity reaction.

TABLE XIV

Prepatent Period, Percent Survivors and Peak Parasitaemia in Post Challange Days

Immunization Group	Percent survivors	Prepatent period (days)	Mean Peak parasitaemia (Mean ± S.D.)	Mean day of death
Group - I (100 ug Ag + 500 ug TDM)	100	9	1.5 ± 0.25	Zero
Group - II (500 ug TDM)	100	6	4.0 ± 1.7	Zero
Group - III (100 ug Antigen)	20	4	20.0 ± 2.9	16.0
Group - IV (Saline Control)	Zero	3	78.0 ± 6.5	8.0

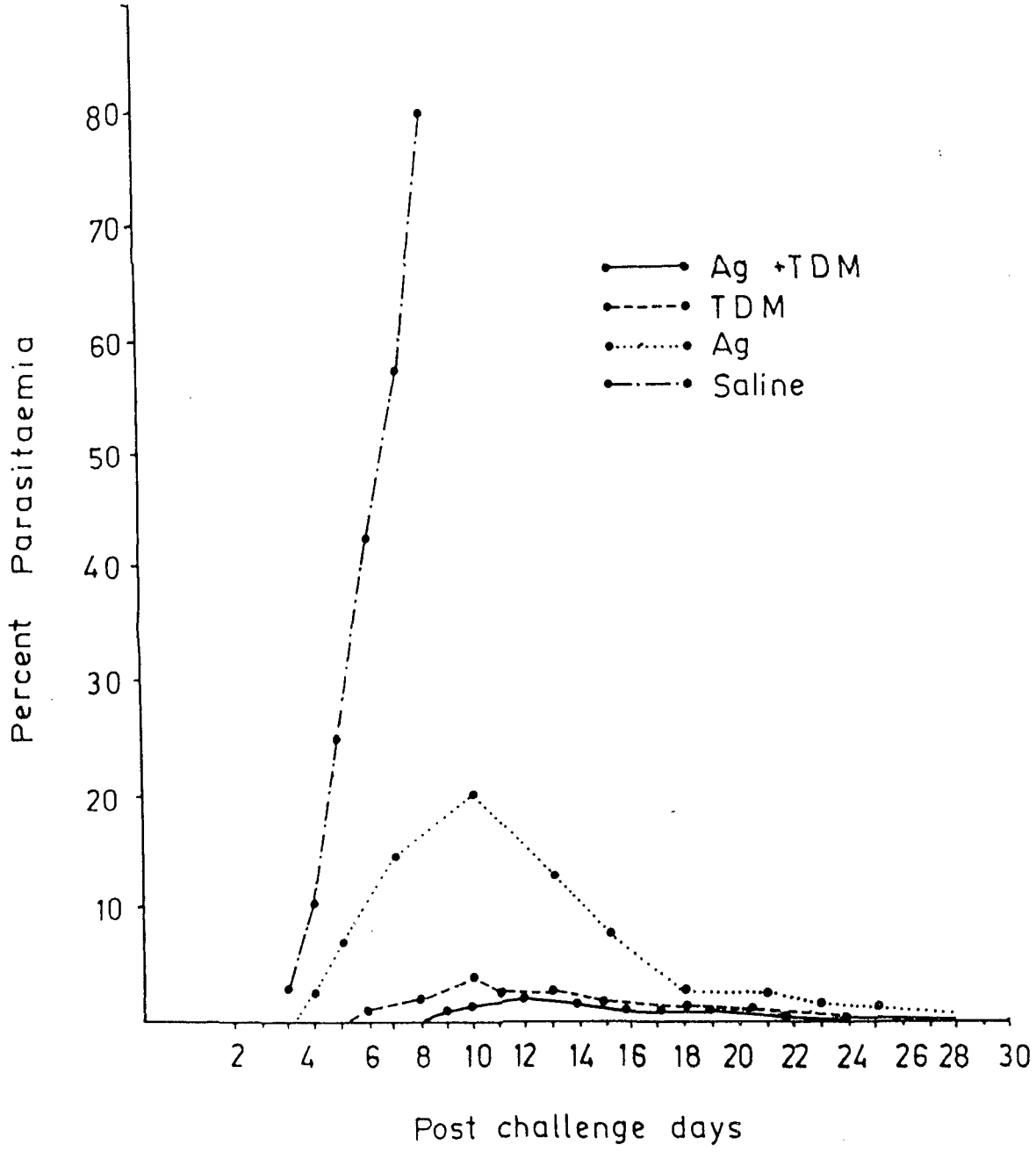
TABLE XV

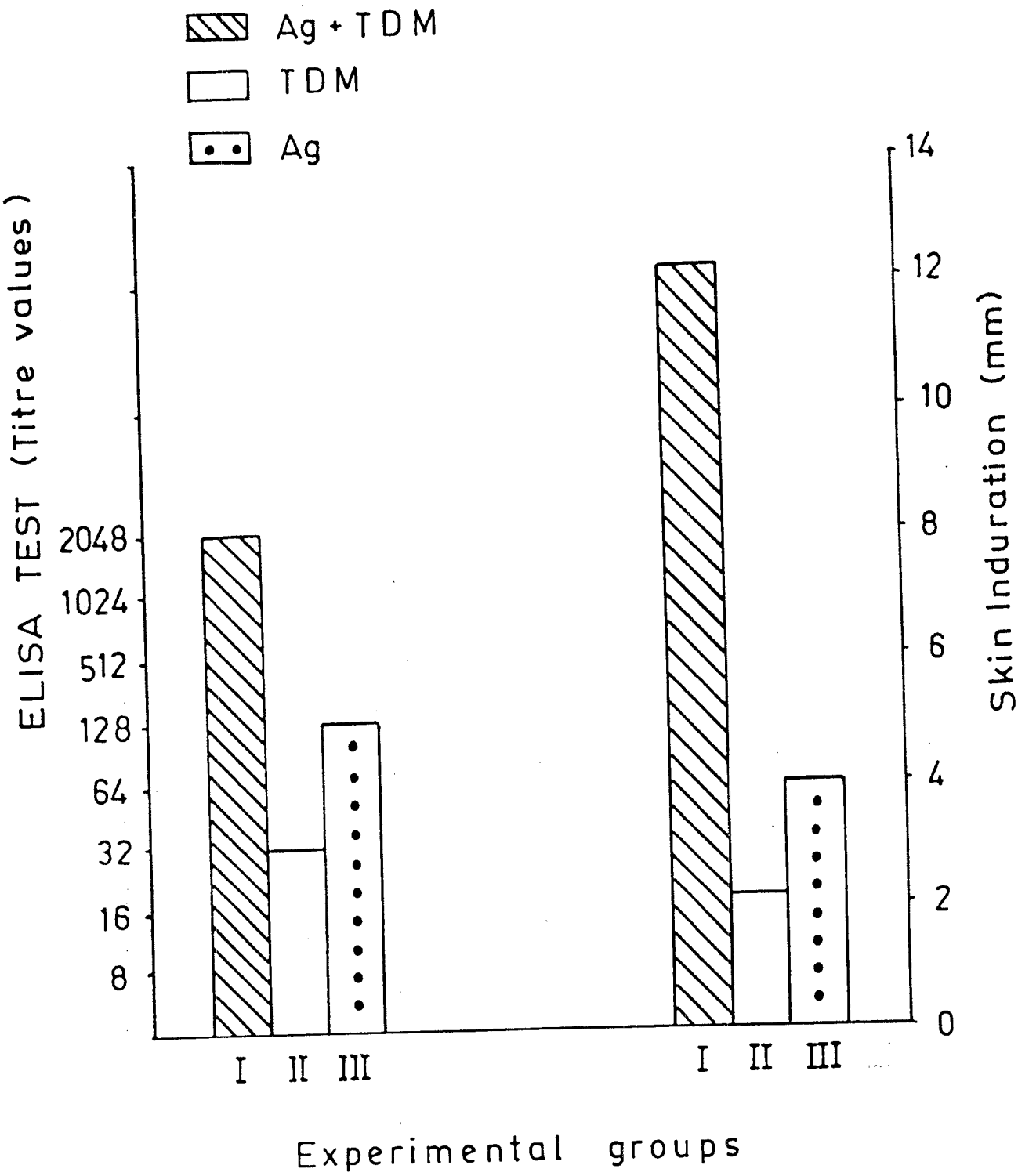
Results of Subinoculation Experiment

Immunization Group	No of mice in each group	No. of survivors	No. of mice subinoculated	No. of infected mice
Group - I (100 ug Ag + 500 ug TDM)	25	25	25	Zero
Group - II (500 ug TDM)	25	25	25	5
Group - III (100 ug Ag)	25	5	5	1
Group - IV (Saline control)	25	Zero	-	-

Ag = *P. berghei* antigen

TDM = 6-6' Trehalose dimycolate


Percent Parasitaemia
80
70
60
50
40
30
20
10
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Post challenge days
Ag +TDM
TDM
Ag
Saline


Ag + TDM
TDM
Ag
ELISA TEST (Titre values)
2048
1024
512
128
64
32
16
8
14
12
10
8
6
4
2
0
Skin Induration (mm)
I II III
I II III
Experimental groups

Similarly cell mediated immune responses were observed only in Ag plus TDM, immunized animals. Animals in this groups developed a well defined skin reaction following skin challenge with antigen. Animal immunized with Ag + TDM showed a skin induration (12.18 mm) (Figure 25).

4.4.10 HISTOPATHOLOGICAL STUDIES

(a) Kidney Histopathology

Hematoxyline and Eosine stained sections from kidney of an immunized animal revealed no alteration in normal architecture. The glomeruli were normal and devoid of pigement. The basement membrane was also normal (Figure 26).

Malarial and hemosiderin pigment was not seen in the kidney tissue. The glomeruli were large and pigmented. The pigment was seen intracellularly within the mesangial cells which appeared proliferated. Some glomeruli showed thickened basement membrane. In glomeruli, lysis of red blood cells was noted. Tubular epithelium was extensively damaged and the accumulation of red blood cells in between the tubules was promient (Figure 27).

(b) Spleen Histopathology

Hematoxylin and Eosin stained splenic section from immunized animals revealed that normal structures were

Figure 26. Immunized kidney section showing glomeruli and tubules. Glomeruli are free from pigment and tubules are normal (H&E x 200).

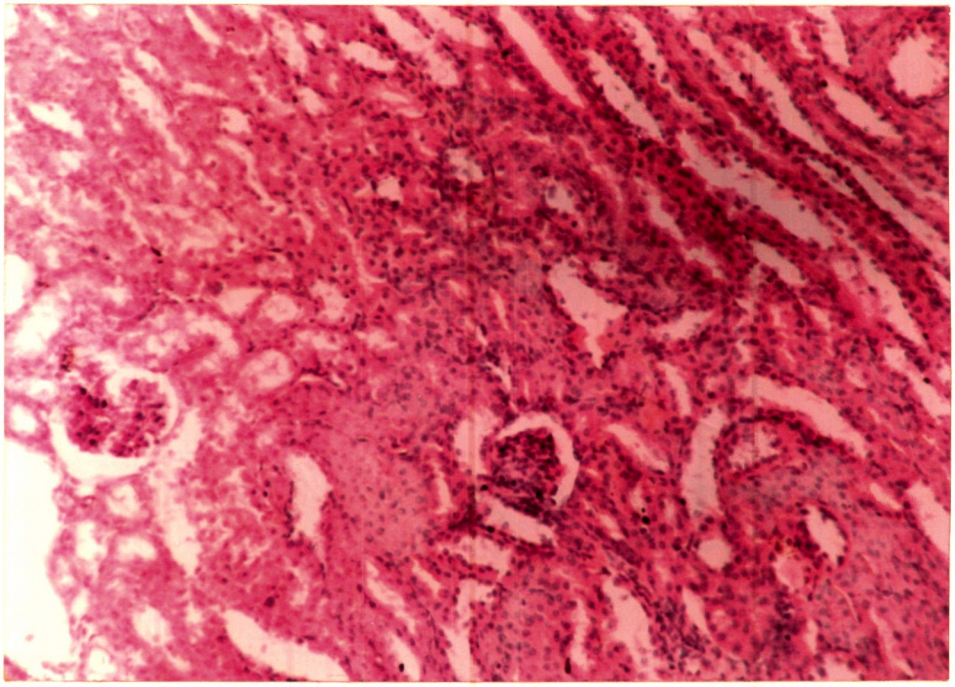


Figure 27. Infected kidney section showing pigmented large glomeruli, tubular spaces are also widened (H&E x 400).

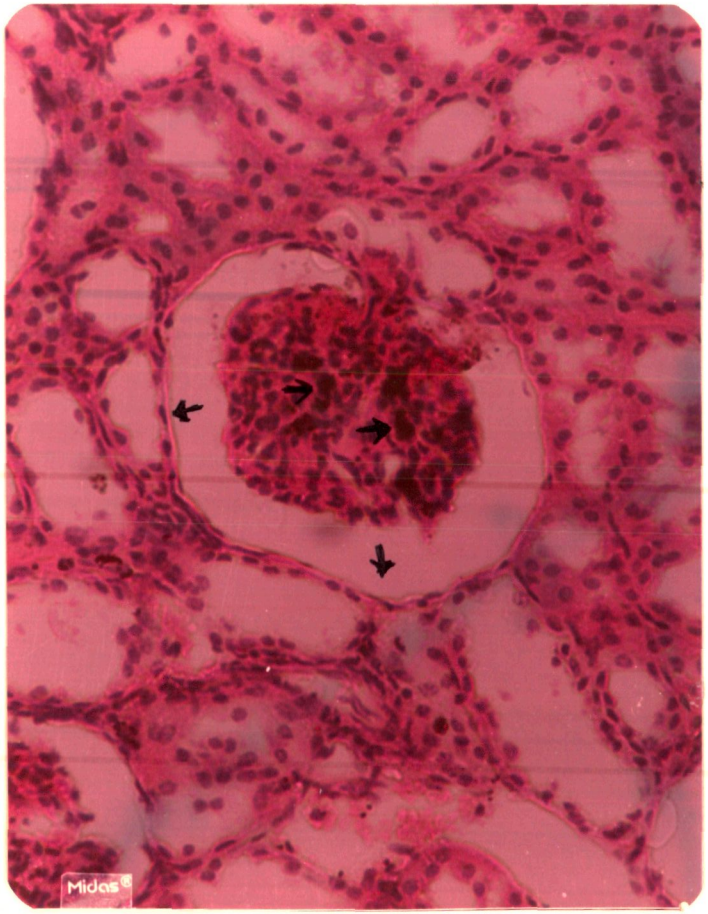

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Figure 28. White pulp of spleen free from pigment (H&E x 200).

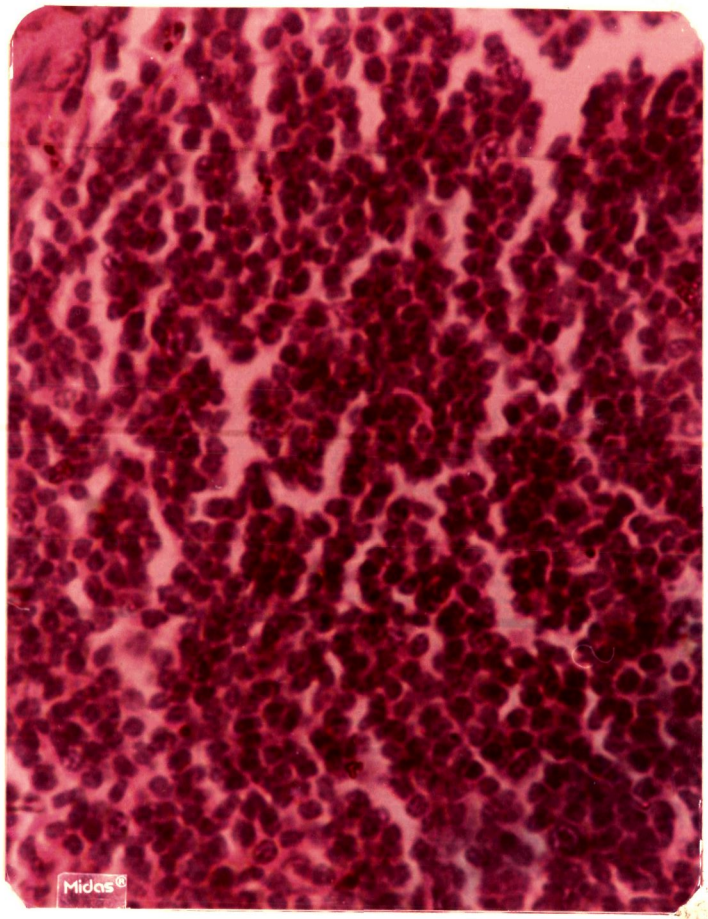
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Figure 29. Spleen section from immunized animal showing white and red kulp and free from pigment deposition (H&E x 200).

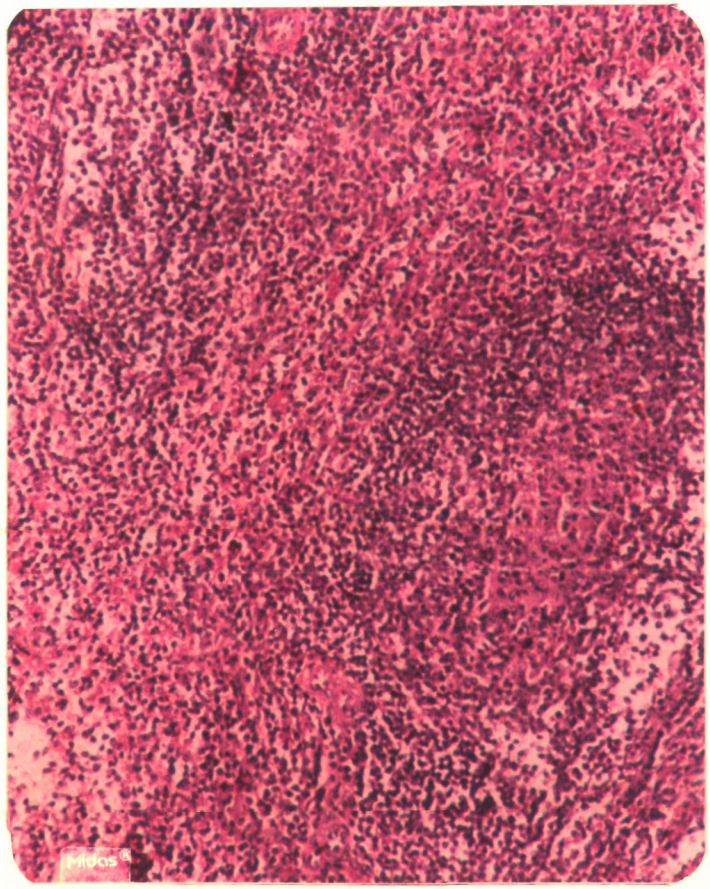
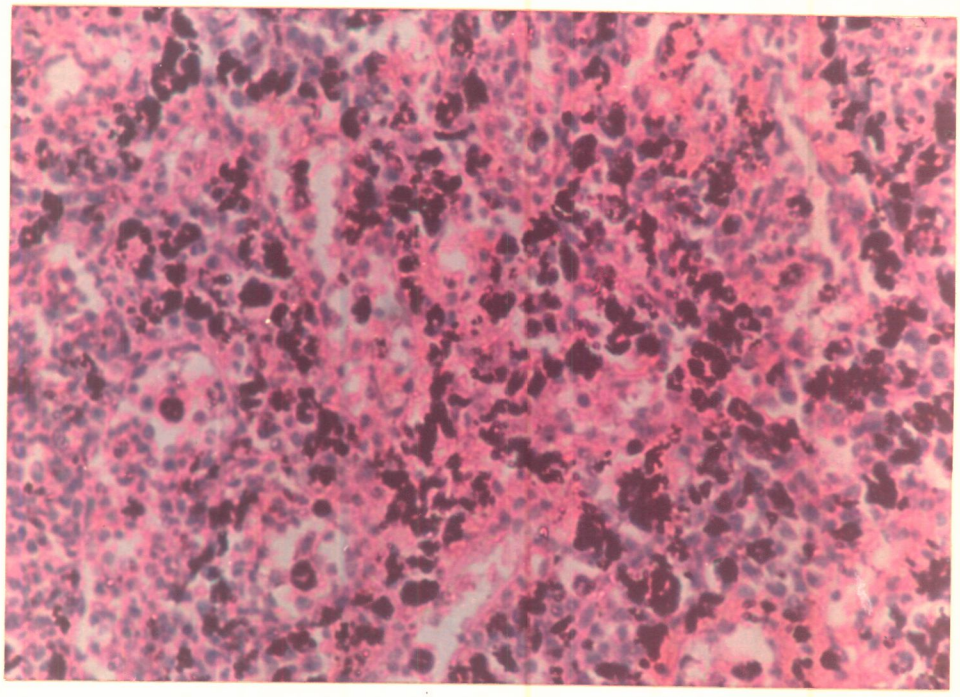


Figure 30. Section revealing the red pulp with clumps of pigment (H&E x 200).



maintained. White and red pulp were found to be free from pigment deposition (Figures 28, 29).

Sections of spleen from infected animals showed a normal splenic architecture. There was marked congestion of splenic sinusoids (Red pulp). Enormous amount of pigment was found in red pulp within swollen and hyperplastic macrophages. Splenic capsule and tuberculae were thickened (Figure 30).

(c) Liver Histopathology

Hematoxylin and Eosin stained sections of liver from immunized animals showed normal architecture. The central veins were devoid of pigment. Kupffer cells were normal and there were no pigment deposits in sinusoids areas (Figures 31, 32). At high parasitaemia (70%) the liver parenchyma showed patchy necrosis in centrilobular region indicating a hepatocellular type of damage in the mouse liver during malaria. Liver sinusoids were extremely dilated during the infection at high parasitaemia. The dilated sinusoids were filled with hypertrophied kupffer cells which contained phagocytosed parasites, and malarial and hemosiderin pigments. Kupffer cells which were swollen and filled with pigment were quite numerous in number. Some central veins and branch of portal veins (Portal triad) showed the presence of pigment within monocytes (Figures 33, 34, 35, 36 & 37).

Figure 31. Section of liver showing central vein devoid of pigments. The sinusoids and hepatocytes are normal (H&E x 200).

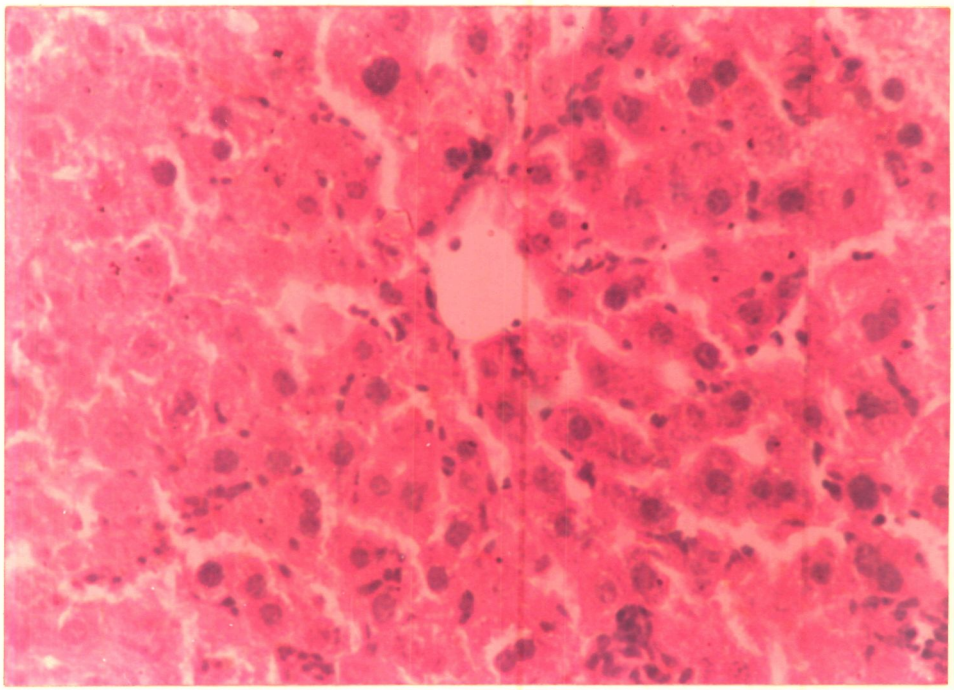


Figure 32. Section from liver showing hepatocytes, sinusoids. Kupffer cells are free from pigment deposition within the sinusoids. Hepatocytes are normal (H&E x 200).

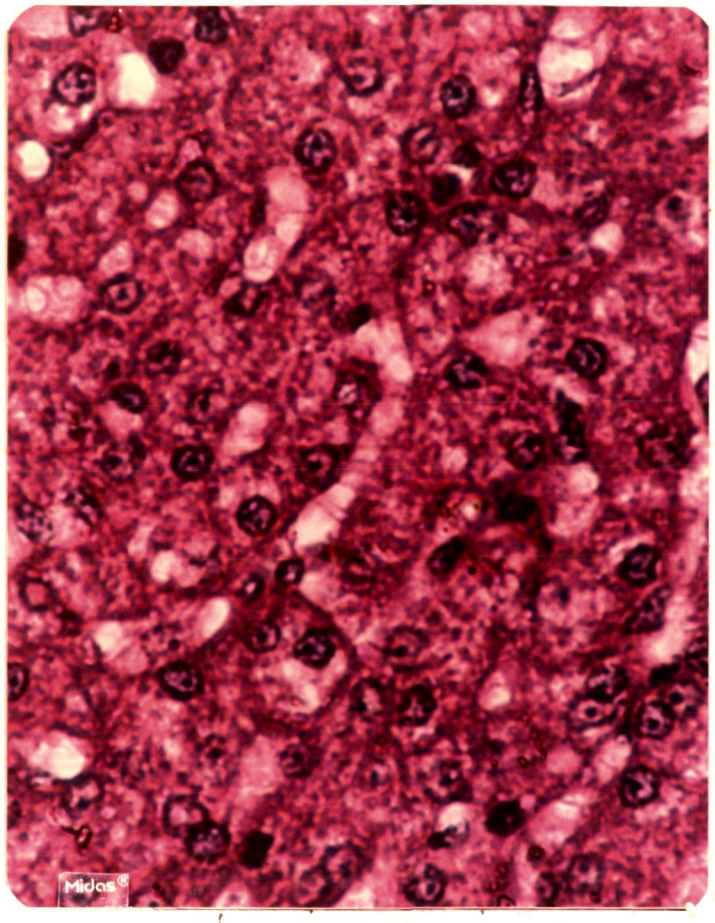

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Figure 33. Section of liver showing deposition of malarial pigments, parasitized erythrocytes and phagocytes within the central vein (H&E x 400).

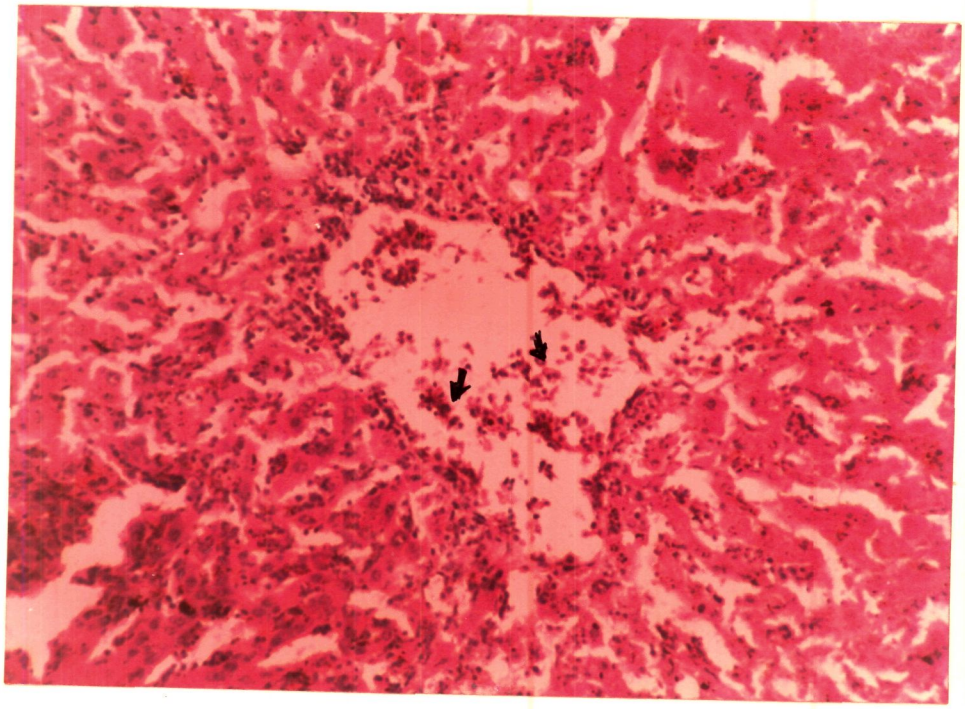
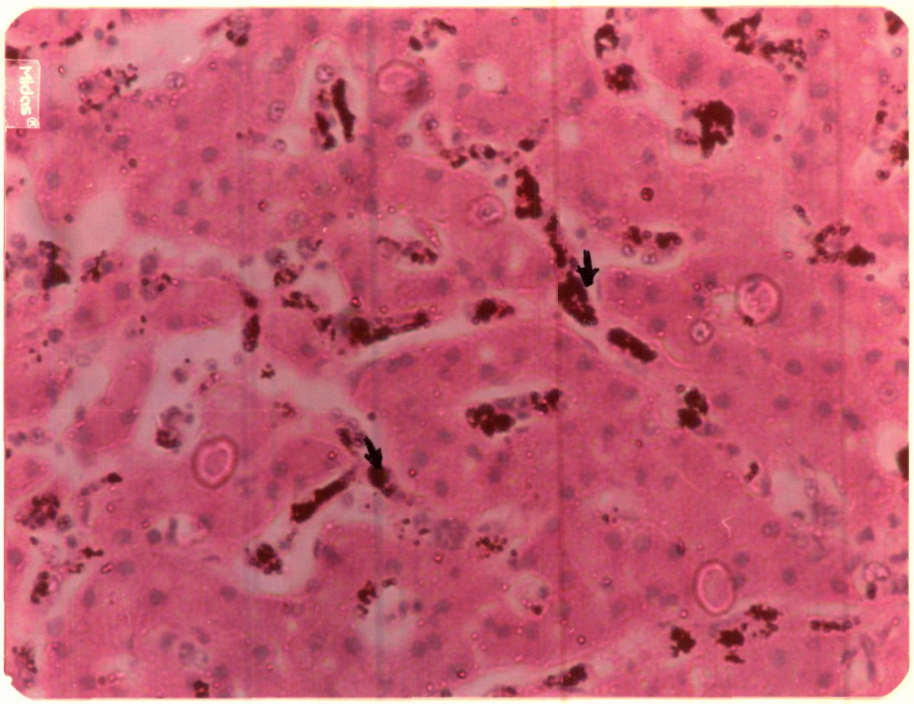


Figure 34. Section from the liver showing cords of hepatocytes separated by sinusoids. The sinusoids show presence of large amount of pigment engulfed within the kupffer cells and macrophages (H&E x 200).

Figure 35. Section from liver showing central vein, hepatocytes and sinusoids. Sinusoids are dilated. Clumps of pigment are seen in central vein and kupffer cells of sinusoids (H&E x 200).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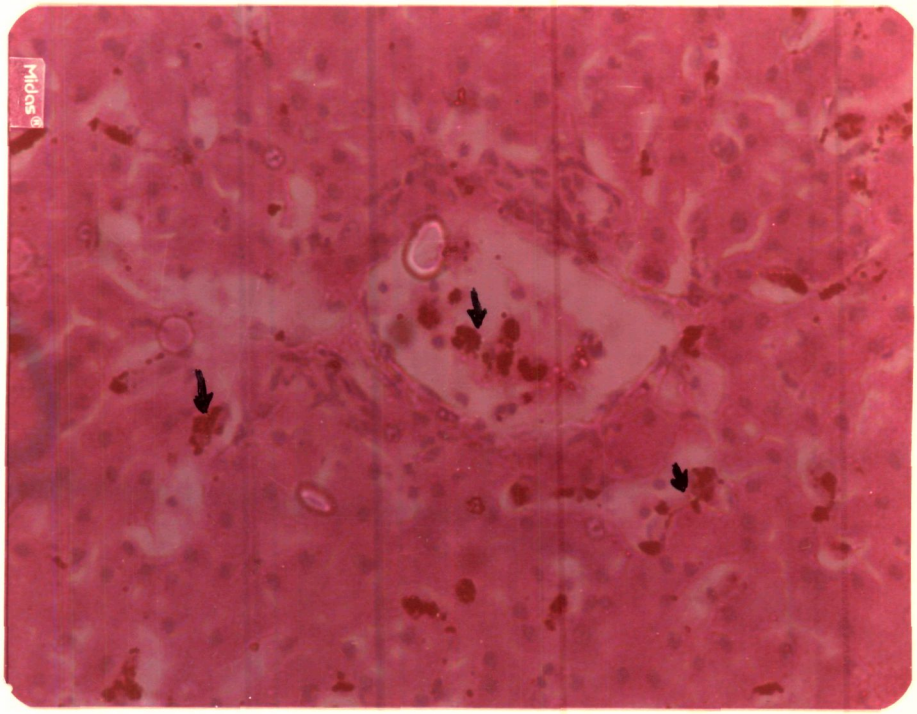
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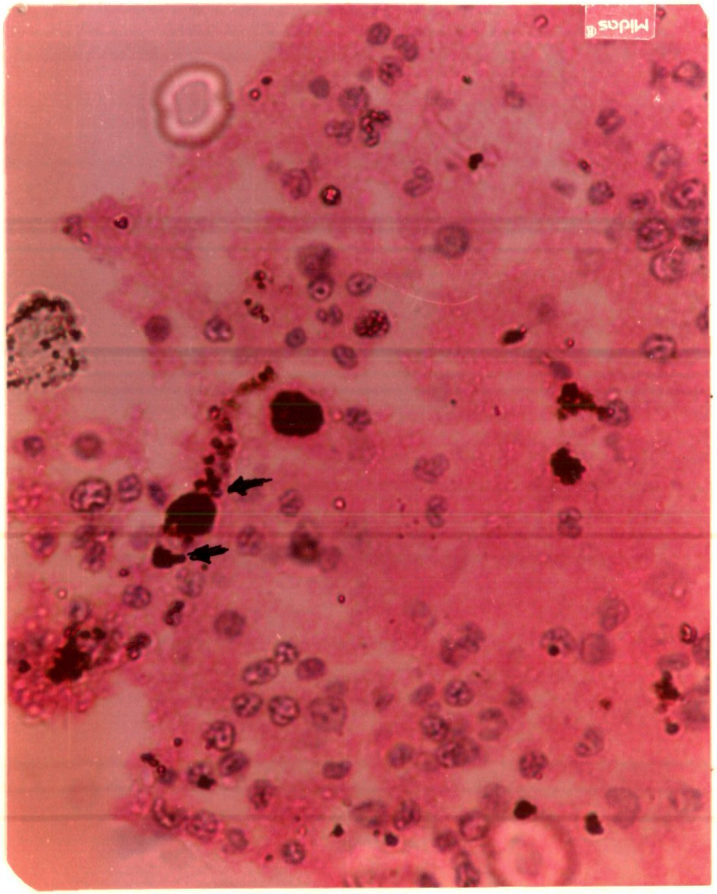
Figure 36. Section showing pigment within kupffer cells and macrophages (H&E x 400).

Figure 37. Section from protal vein showing clumps of intracellular pigment (H&E x 400).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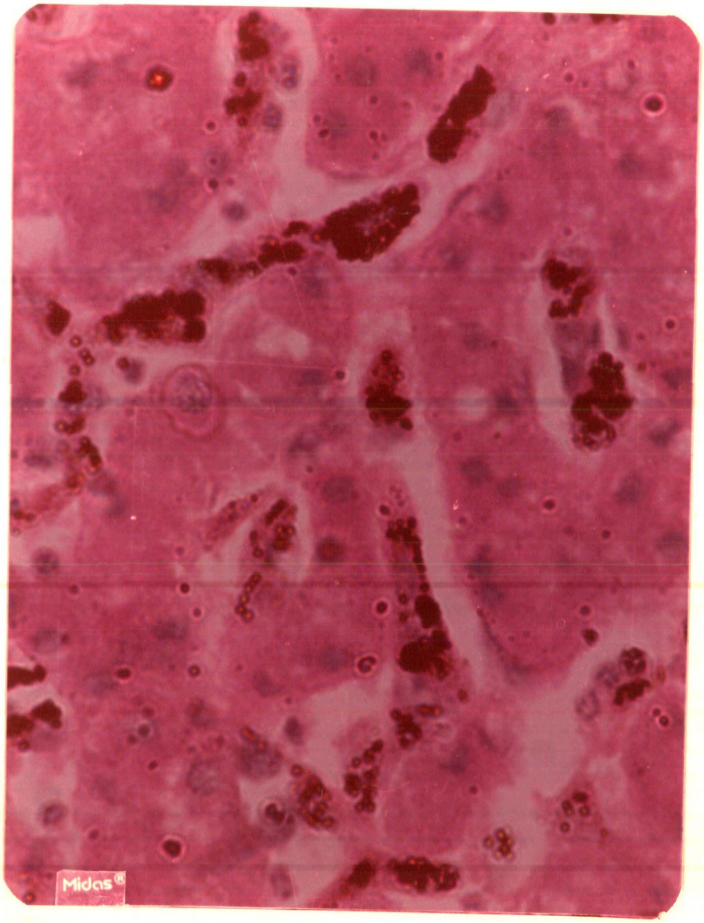
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Malaria has always been one of the major threats to public health in India, as it has been in many other tropical and subtropical countries. The effect of such a debilitating condition on the wellbeing and productivity of a population, already challenged by poverty, malnutrition, and other burdens is, enormous. On account of the resurgence of the disease, malaria research has assumed a high degree of urgency. Research covering different aspects of the disease viz. operational aspects of malaria control, ecology, control of vectors, chemotherapy, drug resistance, serology, biochemical and immunological aspects is being carried out in India and also throughout the World (WHO, 1990; Siddiqui, 1991).

Inspite of enormous amount of literature available on malaria, very limited information exists on the physiological changes produced in the host by the malarial parasites. This deficiency can be ascribed primarily to the fact that biochemical studies on liver and serum of human subjects naturally infected with malaria are often complicated by the presence of concurrent infections and nutritional deficiencies (Desowitz, 1987).

The isolation and description of the rodent malaria parasites Plasmodium berghei by Vincke and Lips (1948) and subsequent studies on experimental transmission of this parasite have permitted the use of the laboratory mouse as a suitable model for experimental studies of mammalian malaria, under

controlled conditions Sadun (1965). In view of the above considerations, studies were set up to determine some of the detectable biochemical changes occurring in mice after infection with P. berghei.

Phenomenon of fatty degeneration in addition to centrilobular necrosis was mainly observed in liver during malarial infection, this was later confirmed by some biochemical studies. In our findings total liver fats were found to increase from 89 percent over control values at 60-70 percent parasitaemia. A similar pattern of increase in hepatic lipid content was also observed in rats, monkeys and humans infected with P. berghei P. knowlesi and P. falciparum, respectively (Rao et al., 1969; Angus et al., 1971; Fletcher et al., 1987 and Bajpai and Dutta, 1990). Accumulation of lipid in liver of animal was also detected by electron microscopy (Fletcher et al., 1987) and by histochemistry (Angus et al., 1971 and Mercado and Von-Brand, 1958). We have also observed lipid deposition in centrilobular region of the liver lobule by histochemical technique. So increase in the level of total lipid in tissue mainly causes hyperlipidaemia at high degree of parasitaemia. There are several reasons for accumulation of fat in liver, which causes fatty liver in animal and human during malaria infection, for example liver plays a decisive role in metabolism and transport of lipids, as well as in the maintenance of lipid levels in the

liver and in blood circulation.

The increase in the level of lipid in albino mice is probably due to a block in the release of hepatic triglycerides in plasma, or perhaps due to disturbed mitrochondrial fatty acid oxidation. This last condition might be due to suppression of lipoprotein lipase activity (Hotez et al., 1984).

Malarial infection was also associated with decreased level of cholesterol content in liver. Seshadri et al. (1983) and Onongbu and Onyeneke (1983) have observed a significant depletion of liver cholesterol content in monkeys infected with P. falciparum. Similarly Rao et al. (1969), and Saxena et al. (1981) also reported decreased hepatic cholesterol content in mouse and rat liver. The present studies have shown that liver cholesterol content in albino mice was depleted significantly by (23.7%) at high degree of parasitaemia. Seshadri et al. (1983) have tried to understand the aetiology of reduction of hepatic cholesterol during P. vivax malarial infection. They have suggested that it might be due to an increased uptake by the infected erythrocytes at high parasitaemia. A similar reason might also be attributed to the decrease of liver cholesterol contents in the albino mice during the course of malarial infection.

Similarly fall in phospholipid levels in the liver

has been observed during the course of malarial infection in mice. Rao et al. (1967) have shown a significant increase in phospholipid levels in the spleen and liver of P. berghei infected mice. They have however (Rao et al., 1969), further reported that phospholipid levels in the infected rat liver were decreased. Sharma et al. (1979) also reported decrease in phospholipid contents in infected mouse liver during P. berghei infection. The phospholipids are essential for the maintenance of membrane structure, the extent of tissue damage with gradual rise in parasitaemia is thus reflected in decreased phospholipid contents.

More significantly our results indicate a remarkable alterations in the rate of lipid peroxidation during chronic infection. The increase was observed to be of the order of (314%) at 60-70 percent parasitaemia. Our results get support from the observations given by previous workers (Saxena et al., 1981; Chauhan et al., 1981). Who have also shown an increased level of lipid peroxidation in the liver of mice during P. berghei infection. Enhancement in the rate of lipid peroxidation following malarial infection is similar to the observations of Bajpai and Dutta (1987). Recently a significant increase in the rate of lipid peroxidation in cerebellum and brain system of mouse following P. berghei infection was shown by Mahdi et al. (1989). Chander and Kapoor (1990) also demonstrated

from their observations that during malarial infection increase in the rate of lipid peroxidation occurs in Mastomys natalenesis infected with Plasmodium berghei. This raised levels of lipid peroxidation are perhaps available due to increased susceptibility of liver to an oxidative damage under the stress of a malarial infection (Sharma et al., 1979).

With the help of biochemical studies, only fatty infiltration in the liver was not observed but a state of hypoglycaemia was also seen (White et al. 1983). This have been due to an altered carbohydrate matabolism. During heavier infections , when liver is likely to be damaged more severely, there would be a state of hypoglycaemia, as has been observed in the present studies. The decrease in carbohydrate content was to be in the order of (78.76%). The reduction observed in carbohydrate content in the liver of albino mice is in agreement with the other reports given by several workers. Homewood and Neame (1980), Saxena et al. (1981) and Clark et al. (1987) have observed depletion in total carbohydrate content of liver in mouse and monkeys infected with P. berghei and P. knowlesi, respectively. Recently, Phillips et al. (1989) and Kawo et al. (1990) have also reported state of hypoglycaemia in humans during P. falciparum infection.

The cause of hypoglycaemia would appear to be due to rapid utilization of available sugar by growing parasites

and also an account of the inabilities of liver to store and produce sufficient amount of sugar. Saxena et al. (1981) and Seshadri et al. (1983) have also tried to explain the physiological cause of reduction of hepatic carbohydrate during malarial infection. They have found that the intraerythrocytic stages of parasite have no carbohydrate reserves and therefore, they obviously consume their nutritional share from host's carbohydrate reserve for their rapid growth and multiplication.

Depletion of total carbohydrate content of liver during malaria infection was also associated with rapid and significant decrease of glycogen and glucose content of liver. Present findings have shown significant depletion of glycogen content in liver of albino mice during P. berghei infection. Liver glycogen content was found to decrease by (84%) at acute infection. Fulton (1939) had examined this problem in P. knowlesi malaria in rhesus monkeys, and reported hypoglycaemia and depleted liver glycogen in the late stages of infection which was mainly due to large consumption of glucose by the parasite. Fletcher (1987) reported that hypoglycaemia is frequently associated with severe malaria at high parasitaemia. Earlier, Sadun et al. (1965) and Srivastava et al. (1984) have reported depletion of glycogen content in liver during malarial infection. Devakul and Maegraith (1958) have observed considerable decrease of liver glycogen in Macaca mulatta infected with

P. knowlesi. Chatterjii and Gupta (1957) have noted similar finding in female rats infected with P. berghei.

However, the glucose contents were also estimated in liver in chronic infections. In our studies we have found depletion in glucose content in liver of mouse by (78.6%) at 60-70% parasitaemia. Fall in glucose content of liver was also observed by Saxena et al. (1981) in Mastomys natalenesis during P. berghei infection. Recently Paul et al. (1991) also reported hypoglycaemia in albino mice infected with P. berghei. Our results in mouse system were similar to those reported in human infections with P. falciparum. Possible mechanism for hypoglycaemia during malarial infection includes accelerated tissue metabolism and increased metabolic requirements of the parasite. Impaired hepatic gluconeogenesis with fatty infiltration causes hypoglycaemia (Filkins and Cornell, 1974). Increase in the concentration of lactic acid in the blood of host causes toxicity leading to tissue damage due to the utilization of glucose through glycolytic pathway by the parasites.

Present findings also revealed decreased amount of total ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Quantitative estimation of total ribonucleic acid and deoxyribonucleic acid confirmed that liver RNA contents decreased by (29%) while DNA decreased by (79%) during severe

infection. The reduction in nucleic acid values as observed in the present studies has not been shown by Rao et al. (1969), who have observed a total increase in nucleic acid contents of liver in chicks during malaria infection. In addition, Pandey et al. (1986) have found no change in nucleic acid contents of liver in P. knowlesi infected monkeys. So it is difficult to ascertain the exact mechanism of reduction of nucleic acid contents of liver observed in our study. Kreier (1980) and Weber et al. (1988), have suggested that nucleic acids are made up of purine and pyrimidine nucleotides. In mammals, purines are both synthesized by denovo and obtained through salvage pathways while malaria parasites are incapable of synthesizing purine by denovo pathway as such and are totally dependent on salvage pathways. Absorption of nucleotides by the parasites from host under such conditions might be one of the reasons for reduction of nucleic acid contents in liver during malarial infection, Nucleic acid contents in infected liver were further assayed by histochemical staining techniques in present thesis.

Marked alteration in the rate of synthesis of protein has been reported in liver tissue of albino mice infected with P. berghei. In the present study, a significant fall in protein contents of liver was found at (60-70%) parasitaemia. This would suggest an appreciable alteration in the protein status of the organs of host. An extensive proteolysis makes readily available

a pool of free amino acids which are needed for rapid proliferation of parasites (Saxena et al., 1981, Fern et al., 1985). Another obvious reason for a decrease in the protein level is the depletion of RNA and DNA contents within the liver of infected animal. In fact, poor availability of nucleic acid could be responsible for lower levels of protein (Chander and Kapoor, 1990).

It is obvious that the interaction of malarial parasites and their hosts gives rise to certain somatic alterations, which may ultimately be reflected in biochemical changes in the body fluids. Therefore, correlation of clinical observations with multiple quantitative determination of the serum components may contribute to the diagnosis and prognosis of this infection, and may even permit an early assessment of the effect of various chemotherapeutic agents (Sadun 1966). The liver damage caused by invading parasites during acute malaria can be more conveniently demonstrated by means of several routine function tests. The enzymes generally used as indicators of liver dysfunction are, glutamic oxaloacetic transaminases (GOT), glutamine pyruvic transaminases (GPT), acid phosphatase and alkaline phosphatase. It has been observed that liver GPT decreased by (22%) and GOT by (42.5%) at 60 to 70 percent parasitaemia respectively. These results are in agreement with the earlier observations made by Lal and Hussain (1978), who

have also observed decreased levels of GPT and GOT activity in the liver of P. berghei infected albino mice, indicating a dysfunction of liver during infection. We have also observed marked alterations in the SGPT and SGOT levels in the serum of infected albino mice. SGPT and SGOT levels was increased by (188% & 84%) respectively. Similar findings were also reported by Sadun et al. (1965), Lal and Hussain (1978) and Khanna et al. (1986) during malarial infection.

During malarial infection, an increase in the number of macrophages, lymphocytes and kupffer cells were also observed in the liver. It was confirmed by estimating the acid and alkaline phosphatase levels in the liver tissues. Biochemical estimations have shown a significant elevation of alkaline phosphatase activity. The elevation in liver alkaline phosphatase was of the order of (75%) at 60-70%% parasitaemia. Increased levels of acid phosphatase activity in the liver was also observed in our studies. It was found to increase significantly by (90%) at high parasitaemia. Saxena et al. (1985) also reported similar findings in various animal models including mice, rats, and Mastomys natalenesis during P. berghei infection. Banyal et al. (1980) also reported acid and alkaline phosphatase activity in monkeys infected with P. knowlesi. They noted altered levels of these enzymes due to the membrane disruption of cytoplasmic organelles which ultimately leads to liver damage.

We have also estimated acid and alkaline phosphatase levels in the serum of infected and control animal groups. Acid phosphatese level in the serum was found to the order of (215%), while serum alkaline phosphatase level was elevated by (99%) during P. berghei infection.

There has been a phenomenal resurgence of malaria as all eradication programmes, based on vector control and chemotherapy measures, have proved to be of limited success (WHO, 1988; Prasad et al., 1990). This situation thus warrants alternative approaches for the control of this infection. In this connection serious efforts are being currently made in India and abroad to explore the possibility of developing vaccine (WHO Tec. Rep Ser. 1975; WHO, 1990; Siddiqui, 1991).

Considering the success of the various vaccine candidates, the use of a potent and safe adjuvant seems imperative for achieving an effective, protective immunity (Siddiqui, 1990). Besides several biochemical studies, we have also made some efforts to assess the role of the adjuvants in immunization and to assess biochemical and pathological events in an immunized host.

The aim of immunization of man and other animals by artificial means is mainly prophylactic against infections. Long lasting, effective immunity results from a controlled stimulation of the immune system by administration of harmless vaccines

rather than from uncontrolled stimulation following a natural course of infection.

The most challenging aspect of malaria research is the isolation of pure parasite material from infected host erythrocytes. For the preparation of malaria parasite from infected blood, the removal of leucocyte cells is considered to be an important step, especially in rodent malaria where there is a large increase in the number of leucocyte during the course of infection (Hickman, 1969). The methods routinely used for the removal of leucocytes and buffy coat is by suspending the washed blood cells in several volumes of dextran solution, or by obtaining them as supernatant following sedimentation (Langer et al., 1967; Zuckerman et al., 1967). Sedimentation velocity centrifugation of washed blood cells on 10% dextran solution (Levy and Chow, 1973) or on Ficoll hypaque solution of density 1.08 gm/ml (Wallach and Conley, 1977) are some of the more commonly employed methods for obtaining the infected RBC free from leucocytes. These procedures are helpful in getting rid of only 75 percent of leucocytes. We used slow speed centrifugation in which about 85 percent of leucocytes were removed. Our results on leucocyte removal compare well with the previous study of Zuckerman et al. 1967. Most efficient method for removal of leucocyte is to pass the washed blood through a column of paper powder as reported by Homewood

and Neame (1976), or through a column packed with equal parts of ∝-cellulose and microcrystalline cellulose (Beutler et al., 1976). We were able to remove 98.5 percent leucocytes by employing this methods. Recently, Mons et al., (1988) demonstrated that the use of a commercially available cell select filter is more effective, rapid and a simpler technique than the use of cellulose powder.

After removal of leucocytes, the next step is lysis of parasitized erythrocytes. In the isolation of parasite, saponin lysis of P. berghei infected erythrocytes has provided a useful method for the recovery of intact parasites. The treatment with saponin results in 100 percent lysis of red blood cells causing the release of free parasites. Siddiqui et al. (1978 b) found that saponin is the most efficient non-ionic detergent for hemolysis, as also for removal of proteins and sialic acid from red cell membrane.

The released parasites can be further separated from erythrocyte contaminant by differential centrifugation, enzymatic digestion, free flow electrophoresis and density gradient centrifugation. But parasites can not be completely separated from erythrocyte fragments by using these procedures. In this study, we were able to obtained comparatively contaminant free P. berghei parasite material by means of Histopaque density gradient centrifugation and slow speed centrifugation. Purification

of parasite was further confirmed when Leishman stained smears of isolated preparation were microscopically observed.

A very important host cell contaminant is the red blood cell material injested by growing parasites. The cell material is usually localized within the phagocyte (Diggs, 1966). Removal of this contaminant has always been a formidiable task. The antigenic material obtained by ultrasonication of parasite (isolated on density gradient centrifugation on Histopaque), showed no host cell contaminant, in immunodiffusion and counter-immunoelectrophoresis, within the sensitivity limits of the tests used for checking such purity. These investigations were successful in obtaining the antigen preparation free from host erythrocyte contaminants. Immunological behaviour of antigen was also checked by employing some serological tests such as immunodiffusion and counterimmunoelectrophoresis.

The isolated antigen and homologus antisera in double immunodiffusion tests on an agarose gel gave at least three precipitin bands. Atleast one band out of these was quite diffused. This band appeared to be made up of more than one precipitin line. Diggs (1966) has carried out immunodiffusion studies on P. berghei antigen-antibodies system. He was also able to detect five precipitin lines in P. berghei antigen using rabbit antiserum. When mouse antisera were used, Seitz (1975) was able to identify four precipitin line. An apparent disparity

in the number of precipitin bands can be explained on the basis of inherent differences in the nature of antiserum, antigenic preparation and the numerous methods employed for extraction.

The antigenic material further, treated on polyacryl amide gel electrophoresis. This investigation also showed that host cell contaminants were not present in the antigenic preparation.

Fractionation of malaria parasite material was carried out for antigenic analysis. In an earlier study, Chavin (1966) used ion exchange chromatography for fractionation of soluble plasmodial extracts. But the results obtained were not fully satisfactory. D. Antonio et al. (1970) carried out fractionation of P. berghei by gel filtration on a Sephadex G-200 column. Fractionation of P. knowlesi antigen was carried out by using Sephadex G-200 column (D' Antonio, 1972) or Biogel A 1.5 (Simpson et al., 1974) but none of the above procedures was found very effective. Although some other workers (Hamberger and Zuckerman, 1976; and Grothaus and Kreier, 1980), who used preparative PAGE for fractionation of P. berghei antigen were not able to fully characterize the parasite material.

Electrophoretic analysis of immunoprecipitated and biosynthetically labelled malaria protein was achieved through sodium dodecyl poly acrylamide gel electrophoresis (Brown et al., 1982). SDS-PAGE technique has also been used by Grothaus et al. (1984) for characterization of P. berghei antigen. They

successfully identified some specific components which were important for the induction of immunity to infection. Wunderlich et al. (1987) have also characterized P. chabaudi parasite and its infected cell host ghost by employing this technique, and they obtained good results by using this method. We have also used SDS-PAGE for fractionation of soluble extracts of P. berghei. The P. berghei antigen resolved in the eleven protein components showing molecular weight in the range of Mr. 12,000 to 1,50,000 daltons (Sharma et al., 1990).

Reports from studies of several workers explained the efficacy of different immunomodulators to protect mice against lethal infections of P. berghei (Khullar and Sehgal 1990, Beuria et al., 1991). In the present studies attempts were made to protect mice against experimental infections of P. berghei following their immunization with purified antigen in combination with TDM (6'6'-Trehalose dimycolate). TDM had been discovered by Bloch (1950) as a glycolipid, secreted on the surface of mycobacterial cells and called cord "factor". Until recently, TDM was tested mainly in oil whereas aqueous suspension of TDM are now preferred, as being more acceptable, and nontoxic. TDM is often used as an adjuvant for natural vaccines, or more frequently for stimulating non specific host resistance leading to antibacterial, antiparasite, antiviral and antitumor activities in experimental models.

In our experiment, the mice which were inoculated with 500 ug TDM alone developed 100% protection against P. berghei. The enhancement of immunity was dependent upon the amount of TDM given to each mouse. One of the advantages of TDM is certainly the long duration of its action. The immunostimulation by TDM is supposed to increase gradually over the weeks. Mice were better protected against Babesia microti 7 weeks after an intravenous injection of 200 ug TDM in aqueous suspension, than after 3 or 5 weeks (Clark 1979). These workers have also reported that mice which were inoculated with 10 ug or 50 ug TDM, intravenously were not protected. Olds et al. (1980) also showed that number of Schistosomulae recovered from mice treated with 200 ug TDM was reduced, but an intravenous dose of 100 ug per mouse was not protective. TDM in a 1% squalane-in-water emulsion was also found to protect mice against Toxoplasma gondii, 4 weeks after intraperitoneal injection (Masihi and Colleagues, 1986). Lederer (1986) also obtained protection in mice by administration of TDM against P. berghei, Toxoplasma gondii, Trypanozoma cruzi, T. musculi, Schistosoma mansoni and Mesocestoides corti. They showed survival rate ranging from 40-100 per cent and the reduction in the infection rate from 30 to 100 percent. Recently, very good percent protection was observed in mouse against P. berghei by administration of 500 ug TDM (Lederer, 1988). Our findings are supported from studies

of Lederer (1986, 1988) observations from our studies also showed that mice which were injected alone with TDM were negative for P. berghei specific humoral and cell mediated immunity. This shows the development of nonspecific immunity against P. berghei by administration of doses of 6-6' trehalose dimycolate. TDM has been shown to enhance non specific immunity against various infection.

TDM can also induce local immunity against an air borne, tuberculosis infection. Pimm et al. (1979) also demonstrated that intraperitoneal injection of TDM causes suppression of an ascitic rat tumor. Similar antitumor activity of TDM in mice has also been reported by Sakurai et al. (1988). Yarkoni et al. (1977) showed that after intraperitoneal administration of TDM into mouse, phagocytosis of L. monocytogenes by peritoneal macrophages increased. On the basis of the facts, Yarkoni et al. (1977) concluded that TDM activates macrophages. Similarly, Kierszenbaun (1984) observed macrophages activation, a few days after intraperitoneal injection of TDM in mouse. These macrophages produced large quantities of H_2O_2 following triggering by phorbol myristate acetate (Lepoivre and Colleagues, 1982). On the basis of various reports there are sufficient evidences to believe that non specific protection, in mouse, following TDM administration, is mainly achieved due to macrophage activation.

A more specific type of protection was also clearly noticeable in our animals which were immunized with antigen TDM mixture. The animals which were immunized with antigen-TDM combination showed a very high level of circulating P. berghei specific antibodies as detected by ID, IHA and ELISA tests. Animals which were immunized with antigen alone were not able to generate such a high level of antibodies. Kumar and Ahmad (1984) in their experiments on immunizations with Ag plus TDM combination showed more protection compared to animals immunized with antigen alone. Sharma et al. (1985) also reported that animals which were immunized with amoeba-TDM combination had higher titres for amoeba specific antibodies. The level of antibodies was comparatively lower in animals injected with amoeba antigen alone. Lederer (1986) also demonstrated that administration of Ag in combination with TDM in mice gave better response against P. berghei infection. Recently, in our laboratory, Pathak (1987) had carried out immunization studies in golden hamsters by inoculating purified Leishmania antigen with TDM. The above study showed that animals which were immunized with Ag-TDM combination showed better protection compared to hamsters immunized with Ag alone.

The cell mediated immune response was generated only in animals treated with P. berghei antigen in combination with TDM. When antigen-TDM combination inoculated mice were checked

for delayed type hypersensitivity reaction, the infiltration of mononuclear cells was fairly evident. The polymorphonuclear leucocytes played an important role as was evident by leucocyte migration inhibition tests in our studies. Kumar and Ahmad (1984) also reported the development of cell mediated immunity in mice immunized with P. berghei antigen-TDM combination. Sharma et al. (1985) observed a strong CMI response in hamsters sensitized by an amoeba - TDM combination. No CMI response was reported in animals immunized with antigen alone. In our studies, no CMI response was observed in TDM and saline injected animals also. These mice failed to generate any humoral and cellular immune responses. But the animals which were injected with Ag-TDM combination showed well defined humoral as well as cell-mediated immune responses which were mainly responsible for 100 percent protection in mice against P. berghei infection.

There are several other adjuvants which provide good protection in mouse infected with P. berghei. Use of glucan as an adjuvant in combination with antigen gave 100 percent protection through development of well defined cellular and humoral immune responses (Kumar and Ahmad, 1985. Maheshwari and Siddiqui, 1989 and Maheshwari and Choudari, 1990). Glucan was also shown to enhance non-specific resistance in experimental animals against various infections but to lesser extent as compared to TDM.

There are numerous reports in the literature demonstrating the protection in mice following the administration of offered Bacillus Calmette Guerin (B.C.G.). It has been successfully used as an adjuvant against large number of infections. Thus BCG has been shown to protect mice against P. berghei (Parashar et al., 1982). Since BCG alone could not afford 100 percent protection in animals, we used TDM in our studies for achieving better protection. TDM seems to enhances host resistance nonspecifically by providing adequate protection to host.

Histopathological studies of various tissues were further carried out to confirm the degree of protection in experimental animals against P. berghei infection. We observed that rodent malaria parasite P. berghei used in this study adversely affects the host vital organs like liver, spleen and kidney, while tissue sections from immunized animal showed no change. Histopathological findings from immunized animals showed no tissue lesions, indicating a normal tissue structure.

Since ages, malaria is a major cause of renal complications (Thayer, 1898). It is reported that malaria impairs the renal function in rodents also (Miller et al. 1967). The histopathological changes recorded in appropriately infected were similar to human malaria (Blainey et al., 1960). Light

microscopy of kidney sections revealed haemoglobin granules in proximal tubules (Boonpucknavig et al., 1973). Tubular damage observed in this study was comparable to glomerulonephritis reported in P. falciparum infection (Iseki et al., 1990). In our study we have also observed large and pigmented glomeruli while immunized animals showed no change at all.

As part of the host defence reaction, the proliferative changes in spleen are commonly observed in all human subjects, primates and rodent malaria (Kreier, 1980; Aikawa et al., 1980). The hyperplasia of white and red pulp as a sign of host defence reaction was also observed. White pulp hyperplasia was seen due to the proliferation of endothelial cells, macrophages and lymphoid elements. Neutrophill infiltration was abdundant especially in the area of necrosis (Taliferro and Mulligan, 1937). Pigment was seen in macrophages, polymorphonuclear leucocytes and parasitized red blood cells. However the section of spleen showed that the white and red pulp were free from pigment deposition in immunized animal.

Hepatomegaly is a very common feature of malaria pathology in humans (Ash and Spitz, 1945), primates (Boonpucknavig et al., 1984) and rodents (Jervis et al., 1968, Bajpai and Dutta, 1987). Malaria parasites metabolize haemoglobin and produce hemozoin pigment, while another type of pigment,

hemosiderin, is produced by lysis of red blood cells. The excessive deposition of these pigment gave dark brown colouration of liver as observed in this study also (Ash and Spitz, 1945, Jervis et al., 1968; Aikawa et al., 1980). Necrosis in centrilobular region of liver is well established in humans (Desowitz, 1967) and monkeys (Rigdon and Thomas, 1942) and in rodent malaria (Jervis et al., 1968). In the present study centrilobular necrosis has also been observed in heavier infections (60-70%). Parasitized and nonparasitized red cells and other infiltrating cells viz. polymorphonuclear cells and lymphocytic cells, were seen in dilated sinusoids of human cases (Rosen et al., 1967) and in primates (Jervis et al., 1968; Gutierrez et al., 1976). Kupffer cells of liver increased tremendously in numbers and became hypertrophied with emulsified malaria pigement and other phagocytosed substances (Taliaferro and Mulligan, 1937, Brito et al., 1962; Aikawa et al., 1980). Liver pathology and histological changes similar to those reported in primate models have also been observed in this study. Necrosis may be caused by obstruction of free flow of blood through the liver sinusoids. Congestion may be a primary factor responsible for the necrosis and such ceribilobular degenerative changes may be due to anoxia (Rappaport, 1963). Out observations also showed that infiltrating cells and kupffer cells occupied the whole of sinsuoidal spaces and reduced the blood flow, thus causing anoxia which may have

a damaging effect on the liver parenchyma which ultimately causes tissue necrosis as observed during P. berghei infection. Such profound biochemical alterations in the liver were also mainly responsible for tissue damage. This was already confirmed quantitatively (by biochemical estimation) and qualitatively (by some histochemical) studies.

We have carried out histopathological studies in immunized animals also by light microscopy. Sections from immunized animal showed that the tissue architecture was found more or less normal and free from pigment deposition.

After completion of immunization, we have assayed some biochemical parameters in the serum of immunized animals in order to confirm whether immunization of animals, could be, if any help in minimizing the biochemical alterations or not. The enzymes generally used as indicators of liver function tests such as SGPT, SGOT, acid phosphatase and alkaline phosphatases were estimated. During infection, the levels of these enzymes were found increased (Lal and Hussain, 1978), but in immunized animals, SGPT and SGOT levels returned to normal. Khanna et al. (1986) also reported similar findings in their rhesus monkeys. When animals were fully recovered from infection, the activities of both, the enzymes, acid and alkaline phosphatase were found normal.

The results of this study suggests that P. berghei infection is of a synchronous nature and can be completely fatal to experimental animals.

Malarial infection causes various structural and functional alterations in different organs of host viz. liver, spleen and kidney. Our results conclude that hepatotoxicity assumes special significance in experimental infection of rodents. The above findings is supported by the fact that liver act as the primary organ for host's homeostasis, and as such various chemical constituents and enzymes of liver including, total lipid, phospholipid, cholesterol, lipid peroxidation, protein, DNA, RNA. liver transaminases and liver phosphatases are invariably altered, significantly. Histochemical studies were also helpful in confirming that massive biochemical changes take place during malarial infection. Similarly, due to liver dysfunction, changes were also observed in serum transaminase and serum phosphatase contents.

Immunization studies were helpful in checking the degree of protection in animals against the infection. Several methods were used for the isolation of P. berghei antigen in pure form. These results are largely based on the various methods which were employed for obtaining antigen preparations in this laboratory. During these investigations, we found that histopaque density gradient centrifugation procedure can be

usefully employed for obtaining comparatively pure antigen preparation. Similarly, sodium do-decyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method was found useful for characterization of P. berghei antigen. The antigen extracts thus obtained were found immunologically active. Successful immunization of albino mice against P. berghei antigen and TDM was carried out. The inoculation of P. berghei antigen alone was capable of generating only a weak humoral and cellular response in immunized animals. Our results indicate that P. berghei antigen must be used in combination with a potent adjuvant for obtaining better results. The animals immunized with Ag-TDM combination showed good humoral and cellular immune responses following challenge with, P. berghei parasites, showing 100 percent protection. In our investigations, injection of TDM alone also showed 100 percent protection. Therefore TDM also provides non-specific resistance to the host as evident from our observations.

The most striking feature in our study was that at high parasitaemia, contents of serum phosphatase and serum transaminase were altered due to liver dysfunction. But in immunized animals these values were near normal. These results further indicate a good correlation between host's resistance to infection and the availability of these enzymes.

The malarial infection adversely affected the host's vital organs such as liver, spleen and kidney. Pathological lesions in the mouse liver infected with P. berghei were identical to those observed in primates, human malaria due to P. knowlesi and P. falciparum infection. Whereas histopathological studies in immunized, protected animals showed normal tissue architecture, free from pigment deposition.

In brief, the various experiments performed during this work showed that infected animals showed massive biochemical alterations in tissue and serum. Similar tissue alteration were also observed in histopathological examination of the infected liver. Such alterations were not seen when similar examination were carried out in immunized animals.

It can be concluded from the results of various experiments that immunization studies might be helpful in minimizing the pathophysiological changes in animals infected with P. berghei.

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